TEEMU TOLONEN

# Molecular Biomarkers and Histopathological Parameters in Prostate Cancer Diagnostics and Prognostics

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ACADEMIC DISSERTATION
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# YHTEENVETO

Eturauhaskoepalat ovat patologin kannalta yksi yleisimmistä näytteistä, joissa diagnosoidaan syöpä. Väitöskirjatyön tarkoituksena oli tutkia eturauhassyöpään johtavia mekanismeja sekä löytää uusia diagnostisia menetelmiä ja ennustetekijöitä.

Eturauhassyöpä on arkkitehtuuriltaan vaihteleva ja yleensä multifokaalinen eli monipesäkkeinen kasvain, jossa eri kasvainfokukset saattavat syntyä toisistaan riippumatta *de novo*. Ensimmäisessä osatyössä tutkittiin ohjelmoituun solukuolemaan eli apoptoosiin osallistuvien biologisten merkkiaineiden BAX ja BCL-2 ilmentymistä potilaskohtaisesti valmistetuissa kudossiruissa, joihin oli valittu eturauhasen normaaleita alueita, liikakasvua, syöpää ja sen esiasteita. BAX:n ilmentyminen oli vähäisintä histologisesti hyvänlaatuisilla alueilla lisääntyen tasaisesti aggressiivista syöpää kohden, ja oli huipussaan alueilla, joissa syöpää tavattiin hermojen ympärillä. BCL-2:n ilmentyminen oli kaksihuippuinen, toinen huippu havaittiin syövän esiasteissa (PIN) ja toinen huonosti erilaistuneessa karsinoomassa. Tulokset viittaavat siihen, että apoptoosin säätely kytkeytyy tiiviisti syövän etenemiseen. Uusi havainto oli, että molempia proteiineja ilmentyi myös jonkin verran syöpää sisältävien eturauhasten mikroskooppisesti normaaleilla alueilla, toisin kuin kokonaan hyvänlaatuisissa eturauhasissa. Löydös viittaa kenttävaikutukseen, joka voi olla eturauhassyövän monipesäkkeisyyden taustalla.

Suomessa todetaan vuosittain 4000 - 5000 uutta eturauhassyöpää, joista suuri osa löytyy oireettomilta miehiltä PSA-testauksen perusteella. Siksi eturauhassyövät ovat nykyään usein pieniä ja hyvin erilaistuneita, joka samanaikaisesti lisääntyneiden näytemäärien kanssa tekee koepalojen histopatologisesta tulkinnasta haasteellista. Toisaalta eturauhasessa tavataan pääasiassa vain yhtä kasvaintyyppiä, adenokarsinoomaa, joka mahdollistaa rutiininomaisesti tehtävien immunohistokemiallisten värjäysten käyttämisen diagnostiikan apuvälineenä. Tutkimme 200 Pirkanmaan sairaanhoitopiin alueella eturauhaskoepaloissa käyneen potilaan välileikkeistä rutiinisti tehtävän kaksoisimmunovärjäyksen vaikutusta diagnostiikan sensitiivisyyteen ja patologin ajankäyttöön. Kaksoisvärjäyksessä neoplastiseen solukkoon sitoutuva AMACR-vasta-aine visualisoitiin sinisellä kromogeenillä ja tyvisoluvasta-aineet p63 ja CK-HMW ruskealla. Tutkimuksessa

todettiin, että rutiinisti tehtävällä kaksoisvärjäyksellä löytyy enemmän syöpäfokuksia kuin nykyisellä käytännöllä, jossa lisävärjäyksiä pyydetään vain osassa tapauksista patologin harkinnan mukaan. Valtaosa (75%) kaksoisvärjäyksen avulla löytyneistä uusista syöpätapauksista oli hyvin erilaistuneita, mutta osa (25%) oli huonosti erilaistuneita. Tulosten perusteella näyttää siltä, että rutiinisti tehtävä kaksoisvärjäys parantaa diagnostiikan herkkyyttä ja nopeuttaa patologin työskentelyä. Pienten fokusten suhteen on kuitenkin syytä muistaa, että varman syöpädiagnoosin tarkkaa alarajaa ei ole määritelty.

Sen lisäksi, että eturauhassyövät ovat muuttuneet, on koepalojen Gleason-luokitus uudistettu vuonna 2005. Tutkimme 247 primaaristi hormonihoidetun potilaan koepaloista uudistetun Gleason-luokituksen ja muiden histopatologisten suureiden ennustearvoa. Kuvannetut näytelasit tutkittiin web-pohjaisella virtuaalimikroskoopilla. Totesimme, että uudistettu Gleason-luokitus on vahvimpia ennustetekijöitä myös primaaristi hormonihoidetuilla potilailla. Sekä pahin koepalakohtainen Gleason-summa että yhdistetty Gleason-summa olivat yhtä hyviä ennustamaan taudin uusiutumista. Lisäksi todettiin uusien suositusten johtavan Gleason-summien yleiseen nousuun ja Gleason-summan 4+3=7 tai yli osoittavan huonontunutta ennustetta. Entuudestaan tiedetään, että eturauhassyöpä leviää eturauhasta ympäröivän kapselin läpi yleensä hermonympärystilassa. Koepaloissa tavattu syöpäkasvu yli kolmen hermon ympärillä osoittautui itsenäiseksi ennustetekijäksi hormonihoidetuilla potilailla. Analysoimme myös kolmen biologisen merkkiaineen (EZH2, Ki-67 ja MCM7) ennustearvoa immunohistokemiallisista värjäyksistä digitaalisella kuva-analyysillä. Näistä solujen jakautumisnopeutta kuvaava Ki-67 indeksi yli 10% osoittautui hyödylliseksi potilaskohtaista riskiä arvioidessa, erityisesti kohtalaisesti erilaistuneissa kasvaimissa (Gleason-summa 7).

Yhteenvetona todetaan, että väitöskirjan osatöissä löydettiin uusia tapoja vastata yhä varhaisemmassa vaiheessa todettujen eturauhassyöpien ja muuttuneiden luokitusohjeiden asettamiin diagnostisiin ja ennusteellisiin haasteisiin.

# Contents

# LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on the following original communications, which are referred to in the text by their Roman numerals.

I Tolonen TT, Tommola S, Jokinen S, Parviainen T, and Martikainen PM (2007): Bax and Bcl-2 are focally overexpressed in the normal epithelium of cancerous prostates. Scand J Urol Nephrol 41:85-90.

II Tolonen TT, Kujala PM, Laurila M, Tirkkonen M, Ilvesaro J, Tuominen VJ, Tammela TLJ, and Isola J (2011): Routine dual-color immunostaining with a 3-antibody cocktail improves the detection of small cancers in prostate needle biopsies. Hum Pathol. In press. DOI:10.1016/j.humpath.2010.12.021.

III Tolonen TT, Tammela TLJ, Kujala PM, Tuominen VJ, Isola J, and Visakorpi T (2011): Histopathological variables and biomarkers enhancer of zeste homologue 2, Ki-67 and minichromosome maintenance protein 7 as prognosticators in primarily endocrine-treated prostate cancer. BJU Int. In press.

IV Tolonen TT, Tammela TLJ, Kujala PM, Tuominen VJ, Isola J, and Visakorpi T (2011): Overall and worst Gleason scores are equally good predictors of prostate cancer progression. Submitted for publication.

# ABBREVIATIONS

| | |
|---|---|
| 2IHC | dual-color immunohistochemical staining |
| 5-ARI | 5-alpha-reductase inhibitor |
| AAH | atypical adenomatous hyperplasia, “adenosis” |
| ADT | androgen deprivation therapy |
| AMACR | alpha-methylacyl-coA racemase |
| AP | alkalic phosphatase |
| ASAP | atypical small acinar proliferation |
| AR | androgen receptor |
| AZGP1 | zinc-alpha-2-glycoprotein |
| BAX | BCL-2-associated X protein |
| BCH | basal cell hyperplasia |
| BCL-2 | B-cell lymphoma 2 protein |
| BPH | benign prostatic hyperplasia |
| CaNE | normal epithelium in cancerous prostate |
| CGS | compound Gleason score |
| CK34βE12 | high molecular weight cytokeratin |
| CK-HMW | high molecular weight cytokeratin |
| COX-2 | cyclo-oxygenase-2 |
| CPC | cores positive for cancer |
| CRPC | castration-resistant prostate cancer |
| cT-stage | clinical tumor stage |
| DAB | diaminobenzidine |
| DRE | digital rectal exam |
| ENUP | European Network of Uropathologists |
| ERG | ets-related gene |
| EZH2 | enhancer of zeste homologue 2 |
| GPC | greatest percentage of cancer in a single biopsy core |
| GS | Gleason score |
| H&E | hematoxylin and eosin -staining |

| | |
|---|---|
| HGPIN | high-grade prostatic intraepithelial neoplasia |
| HIF-1α | hypoxia inducible factor-1 alpha |
| IAP | inhibitor of apoptosis |
| IHC | immunohistochemistry |
| ISUP | International Society of Urological Pathology |
| Ki-67 | cell proliferation marker (also known as MIB-1) |
| LBP | local binary pattern |
| LGPIN | low-grade prostatic intraepithelial neoplasia |
| LHRH | luteinizing hormone-releasing hormone |
| MCM7 | minichromosome maintenance protein 7 |
| MTA1 | metastasis-associated protein 1 |
| MUC1 | mucin 1 |
| NAIP | neural apoptosis inhibitor protein |
| OGS | overall Gleason score |
| p16 | protein 16 |
| p53 | protein 53 |
| p63 | protein 63 |
| PAP | prostatic acid phosphatase |
| PIA | proliferative inflammatory atrophy |
| PIM1 | serine/threonine-protein kinase |
| PNI | perineural invasion |
| PSA | prostate specific antigen |
| pTEN | phosphatase and tensin homolog |
| SPINK1 | serine peptidase inhibitor, kazal type 1 |
| TAUH | Tampere University Hospital |
| TMA | tissue microarray |
| TMPRSS2 | transmembrane protease, serine 2 |
| *TMPRSS2:ERG* | gene fusion specific for prostate cancer |
| TNM | tumor, lymph nodes and metastasis |
| TPC | total percentage of cancer |
| TURP | transurethral resection of prostate |
| VEGF | vascular endothelial growth factor |
| WGS | worst Gleason score |
| WHO | World Health Organization |

# ABSTRACT

A prostate needle biopsy is one of most common types of samples that pathologists use to diagnose a malignancy. The main aims of this study were to investigate the mechanisms leading to multifocal prostate cancer and to identify new diagnostic methods and prognostic markers for prostate needle biopsies.

Prostatic adenocarcinoma is an architecturally diverse tumor that typically shows multifocal growth and several grades. Multiple tumor nodules may arise either from microscopic infiltrations of the primary cancer focus or are more likely *de novo*. Apoptosis is also involved in the development of prostate cancer. We studied apoptosis regulation in general, with a special emphasis on its role as a possible mechanism underlying multifocality. All essential histological features of the prostate specimen were sampled using tissue microarrays. The expression of two important regulators of apoptosis, BAX and BCL-2, was immunohistochemically studied in 1182 foci representing morphologically normal epithelium of cancerous prostates, benign prostatic hyperplasia, prostatic intraepithelial neoplasia, adenocarcinomas (grades 1 to 5), and capsular perineural invasions. BAX expression steadily increased in more malignant phenotypes, while BCL-2 expression was biphasic, with overexpression observed in prostatic intraepithelial neoplasia (PIN) and in poorly differentiated cancers. Interestingly, both proteins were overexpressed in a subset of morphologically normal foci of cancerous prostates, but not in controls or in cases of hyperplasia. This observation is consistent with the field-effect theory, which may explain the multifocality observed in prostate cancer.

Currently, 4000-5000 patients are diagnosed with prostate cancer in Finland annually, most of whom are identified by frequent prostate specific antigen (PSA)-testing of asymptomatic men. Prostate cancer is diagnosed from needle biopsies, and an increasing number of biopsies with small foci and atypical features are a diagnostic challenge for pathologists. The vast majority of prostate cancers are adenocarcinomas, making routine application of immunohistochemistry possible. The impact of routine, dual-color immunostaining (2IHC) on diagnostic sensitivity and pathologists' workloads was examined using interval sections from 200 prostate needle biopsies. In our 2IHC staining protocol, the neoplastic epithelium was visualized with a blue chromogen, and the basal cells were stained brown. Our

results suggest that routine 2IHC leads to increased sensitivity and efficiency in cancer detection. Most of the small cancers detected with the aid of 2IHC were low-grade (75%), but a number of very small foci with poorly differentiated cancer (25%) were also observed. No minimal criteria for a definitive cancer diagnosis, however, have been established.

In addition to the biological changes observed in more recently diagnosed prostate cancers, the Gleason classification system of needle biopsies underwent major refinements in 2005. We studied the prognostic value of the modified Gleason score (GS) and other histopathological features of needle biopsies from 247 patients who were primarily endocrine-treated. Hematoxylin and eosin (H&E)-stained slides were scanned, and the evaluation of the digitalized slides was performed using a web-based virtual microscope. Our results show that the modified GS is one of the strongest independent prognostic factors for primarily hormone-treated patients. Moreover, we show that both the worst GS in a single biopsy and the overall GS performed equally well as prognostic factors. In addition, the new recommendations lead to an increase in GSs in general, and the use of modified the GS may have shifted the cut-off between low- and high-grade cancer from GS 6 vs. 7 to GS 3+4 vs. 4+3. Local spreading of prostate cancer usually occurs through the prostatic capsule in the perineural space. More than three foci of perineural cancer invasion in the biopsies independently predicted recurrence of the disease in hormone-treated patients. In addition, the expression of three promising biomarkers predictive of survival (EZH2, Ki-67, and MCM7) was analyzed with the aid of digital image analysis. In patients with GS 7, a Ki-67 labeling index greater than 10% distinguished patients with an early PSA recurrence.

In conclusion, new diagnostic applications and prognostic tools for prostatic needle biopsies were identified. These methods will hopefully be useful to pathologists in the PSA era.

# INTRODUCTION

In the past two decades, prostate cancer has gained special public interest. The general awareness of the disease has emerged due to its high incidence and prevalence and also because of celebrities with prostate cancer (e.g., Francois Mitterrand, Frank Zappa and Robert De Niro).

The overall incidence of prostate cancer in Finland is approximately 100 cases per 100 000. The incidence begins increasing slowly at 45 years of age, and maximal incidence is noted between 70 and 74 years. However, even more new cases are noted in the age range of 80-84 if data are age-adjusted (1154 per 100 000) (Finnish Cancer Registry, Cancer Statistics at www.cancerregistry.fi, updated on 07.12.2010).

Between the years 1980 and 2008, the prostate cancer incidence in Finland increased four-fold from approximately 1000 annual cases in the early 1980s to 4215 cases in 2008. In the early 1990s, the number of new prostate cancers exceeded that of registered basal cell skin carcinomas, which is usually considered the most common malignancy. In the mid-2000s, the incidence of prostate cancers exceeded that of breast cancer. Over the past few years, the incidence of prostate cancer has slightly decreased, while the number of new breast cancers increased. As a result, breast cancer was once again the most common cancer in Finland in 2008 (Finnish Cancer Registry, Cancer Statistics at www.cancerregistry.fi, updated on 07.12.2010). A similar decrease in the incidence of prostate cancer was observed ten years earlier in the U.S., where testing of prostate specific antigen (PSA) levels was first introduced (Quinn and Bapp 2002).

There are at least three explanations for the observed increased incidence of prostate cancer. Two of these include increases in cancer awareness and better diagnostic sensitivity in the early 1990s, such as high-resolution trans-rectal ultrasound devices and the increased use of needle core biopsies (Cremers et al. 2010). The most evident explanation for the increased incidence of prostate cancer, however, is PSA testing of asymptomatic men (Cremers et al. 2010).

Based on clinical follow-up-studies and autopsy specimens, most prostate cancers are indolent. In 2008, however, prostate cancer was the second leading cause of cancer-related deaths in Finland and in the U.S Furthermore, the annual mortality due to prostate cancer increased two-fold between 1980 and 2008 in Finland, from approximately 450 to 814 deaths per year. The international trend of prostate cancer-specific mortality is slowly rising to approximately 30 deaths per 100 000 people in Western countries (Quinn and Bapp 2002). Age-adjusted mortality for prostate cancer in Finland has decreased in the past few years to approximately 15 per 100 000 males (Finnish Cancer Registry, Cancer Statistics at www.cancerregistry.fi, updated on 07.12.2010).

The etiology of prostate cancer remains unknown. The regulation of both normal prostate function and prostate cancer is androgen-dependent, and the benefits of androgen withdrawal therapy were known seventy years ago (Huggins and Hodges 1941). There is also a strong genetic predisposition, as evidenced by familiar clustering of the disease and twin studies (Steinberg et al.1990, Grönberg et al.1997, Lichtenstein et al.2000). Interestingly, most prostatic adenocarcinomas are multifocal (Qian et al. 1997), consistent with the cancer field effect theory. There is cumulative evidence suggestive of field cancerization of the prostate, including gene/protein expression abnormalities and epigenetic changes detected in benign-appearing epithelium adjacent to cancerous tissue (Chai and Brown 2009, Nonn et al 2009).

There are three primary forms of treatment for prostate cancer: active surveillance, curative treatments, and endocrine control of cancer (Heidenreich et al.2010, Mottet et al. 2011). Surgical treatment of well-differentiated cancer decreases cancer-specific mortality and morbidity when compared to watchful waiting (Bill-Axelson et al. 2005). However, a significant proportion of men diagnosed with low-grade cancer can have a favorable long-time prognosis (Albertsen et al. 2005b) and are at risk of being overtreated. One treatment option for patients with well-differentiated local cancer is active surveillance, which is currently chosen by approximately 10% of men (Cooperberg et al. 2007). In contrast to watchful waiting, active surveillance refers to intense follow-up and early intervention, if required. In a review by Dall'Era et al. (2008), the following conservative criteria were recommended to identify suitable candidate patients for active surveillance: PSA value < 10 ng/ml,

stable PSA kinetics, Gleason score ≤ 6, cT1-T2a, and a low-volume cancer based on at least 12 needle biopsies. In the studies reviewed by Dall'Era et al., commonly used criteria for a low-volume cancer were: i) demonstrating fewer than 1/3 positive cores and ii) less than 50% involvement in a single core (Dall'Era et al. 2008). Preliminary studies of active surveillance indicate that the outcome of these patients is not compromised (Klotz 2006). However, because the natural history of prostate cancer is often delayed (Johansson et al. 2004), longer follow-up times are required. Intent-to-cure treatment options for local cancer are radical prostatectomy, external-beam radiotherapy or brachytherapy. Patients with locally advanced cancer benefit from 3-year adjuvant androgen deprivation therapy (ADT) (Heidenreich et al. 2010). Metastasized disease is hormonally treated with luteinizing-hormone releasing-hormone (LHRH) agonists as a first-line option (Mottet et al. 2011). Although almost all patients respond to ADT, the disease eventually progresses if the patient lives long enough (Palmberg et al.1999). In such castration-resistant disease, the use of docetaxel may prolong life for a few months and is encouraged (De Dosso et al. 2008, Mottet et al. 2011). In localized disease, hormone-therapy is not associated with better long-term survival (Lu-Yao et al. 2008). The treatment chosen for a given patient depends on the patients' expectations and clinicians' recommendations based on prognostic factors, the most important of which are tumor, nodes and metastasis (TNM)-stage, PSA, and Gleason score.

Chemoprevention of prostate cancer is still under debate. In preliminary trials of benign prostatic hyperplasia (BPH), the use of finasteride was associated with a reduced incidence of prostate cancer. Since then, several clinical trials have been designed to specifically address this issue. Finasteride and dutasteride are inhibitors of 5α-reductase (5-ARI), which converts testosterone to the more potent hormone dihydrotestosterone. In the Prostate Cancer Prevention Trial, finasteride was shown to reduce the risk of prostate cancer by 25% at the end of the seven-year follow-up (Thompson et al. 2003). Subsequently, similar results were observed in the REDUCE Trial with dutasteride (Andriole et al. 2010). In human cancer cell lines, finasteride induces apoptosis in a dose-dependent manner via the BCL-2 and BAX/caspase family of proteins (Golbano et al. 2008). Other preventive agents currently awaiting validation include cyclo-oxygenase-2 (COX-2) inhibitors, statins, and dietary supplements (reviewed by Rittmaster et al. 2009).

Frequent testing of prostate cancer has led to earlier detection. The median age at the time of detection is now a few years lower than before the PSA screening era (Cremers et al. 2010). Cancers detected from elevated PSA levels, are smaller and of lower stage and grade than their clinically detected counterparts (Laurila et al. 2009, Cremers et al. 2010) and may be overlooked by needle biopsies (Wolters et al. 2010). Many of the small well-differentiated cancers restricted to one biopsy core are currently treated by active surveillance, which consists of follow-up PSA tests, digital rectal exams (DRE) and repeated biopsies. Due to the aforementioned issues, pathologists are increasingly exposed to prostate needle biopsies. The diagnosis of very small cancer is subjective, and even the definition of a lower limit of cancer is difficult (Van Der Kwast et al.2010). However, small-foci cancers present in biopsies often represent clinically significant (GS≥7, bilateral, multifocal or >0.5 cc) disease in radical prostatectomy specimens (Boccon-Gibod et al. 2005, Montanari et al. 2009). Therefore, the accurate detection of all cancerous foci in prostate needle biopsies is important. In addition, it affects prognostic parameters, such as the number of positive cores, and thus may directly influence the treatment choice.

At the same time that cancer detection became earlier, the Gleason scoring system went through several refinements (Epstein 2000, Epstein et al. 2005), which paradoxically led to increased Gleason scores (Albertsen et al. 2005a, Thompson et al. 2005). Thus, a well-differentiated prostate adenocarcinoma today is not the same as it was two decades ago (e.g., GS 6 versus 4), which may interfere with the results from long-term follow-up studies. In addition, the prognostic impact of the modified Gleason scoring system needs to be further evaluated.

Clearly, there are many challenges for prostate histopathology, and new diagnostic aids and prognostic tools are needed.

# REVIEW OF THE LITERATURE

# 1. Histopathological acinar findings in the prostate gland

## 1.1 Introductory gross anatomy and physiology

The prostate gland surrounds the prostatic urethra and the urinary bladder neck distal to the bladder. Like the heart and *uterus*, the prostate is "upside down", with its *apex* facing downwards and its *base* facing upwards (Myers et al. 2010). In 1968, McNeal proposed a practical concept of dividing the prostate into central, transitional, and peripheral zones (McNeal 1968). Anatomical models with lobar architecture have also been proposed (Tisell and Salander 1975). However, most normal prostates lack lobar architecture (Myers 2000). In more modern prostate nomenclature suggested by urologists and anatomical pathologists at the Mayo Clinic, the use of the term "central zone" is considered problematic because it can become confused with the central part of gland (Myers et al. 2010). The use of the term "lobe" is also discouraged (Myers et al.2010).

Seminal vesicles and the *ductus deferens* fuse to form two ejaculatory ducts, which enter the prostate at the base. These ducts continue in the midline, anterior to the peripheral zone, to the verumontanum, where they open to the urethra (Myers et al. 2010). The apex, basis and zones are important to distinguish because of the different cancer frequencies, cancer grades, and surgical implications associated with these regions (Cohen et al. 2008).

Functionally, the prostate gland is an additional reproductive organ that contributes to the viscosity of the semen by secreting enzymes, including PSA, prostatic acid phosphatase (PAP), and alkalic phosphatase (AP). PSA is a chymotrypsin-like glycoprotein that slowly hydrolyzes peptide bonds, thereby liquefying semen. Some of the enzymes secreted by the prostate can be detected in circulating blood (e.g., PAP, AP and PSA) and used for diagnostic purposes (Stamey et al.1987, Barichello et al.1995, Strohmaier et al. 1999).

## 1.2 Normal prostate histology

The peripheral part of the prostate gland consists of small glands (“acini”) that drain to ducts, which fuse to form bigger ducts. These eventually drain into small openings of the prostatic urethra. Benign prostatic acini are lined with a double-layered epithelium (McNeal JE 1988). The luminal compartment of the epithelium consists of cuboidal to columnar cells with abundant, clear cytoplasm, the amount of which depends on the secretory activity of the gland (Figure 1). The outermost layer consists of flat, triangular cells with small nuclei and scarce cytoplasm, the so-called basal cells. There is zonal variation of the normal morphology. For example, the morphology of central zone glands is between that of the peripheral glands and seminal vesicles.

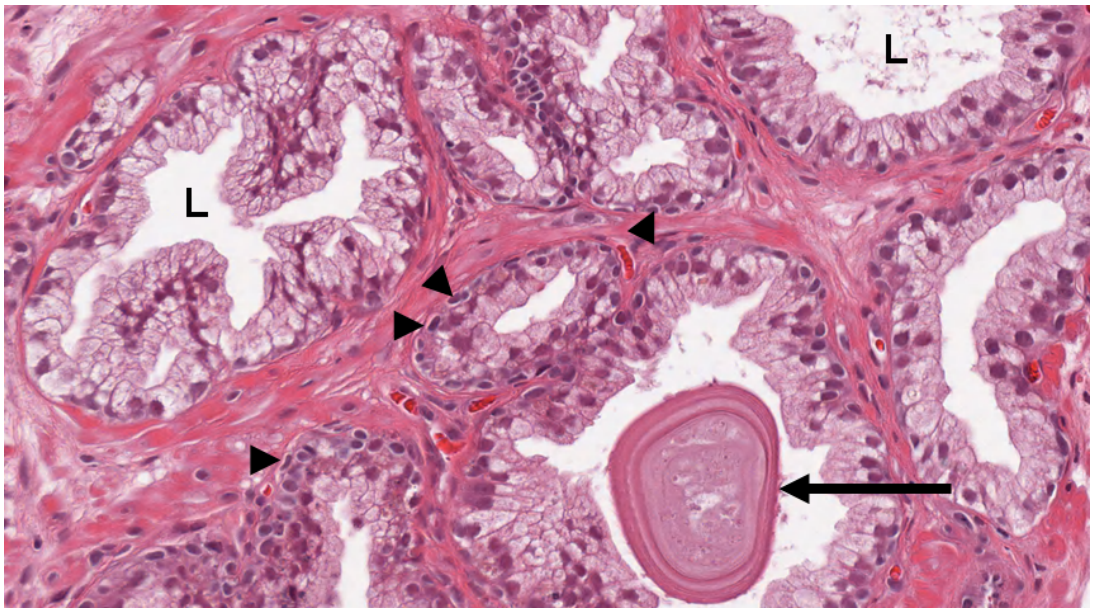


Figure 1. Benign histology of the prostate. Luminal (L) epithelial cells are columnar and have clear, abundant cytoplasm. The glands are lined by basal cells (arrowheads). Occasionally, psammoma-like bodies referred to as *corpora amylacea* may be observed inside the glands (arrow). H&E staining (200 x).

## 1.3 Benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is a common cause of prostate enlargement that leads to clinically significant bladder outflow obstruction in approximately 40% of men (Djavan 2010, Roehrborn 2011). Most often, the histological changes associated with BPH are noted in the periurethral region (Myers 2000). The first change consists of an increased amount of smooth muscle and less elastic tissue than the normal stroma. These events are followed by hyperplasia of the periurethral glands (Rosai 1996). The glands are often dilated and have a cytologically benign-appearing epithelium with inconspicuous nucleoli (Rosai 1996).

The diagnosis of BPH is often clinical and based on symptomatic prostatism, DRE, serum PSA value, free/total PSA %, and findings on transrectal/transabdominal ultrasound. Treatment of symptomatic BPH is now primarily through pharmaceuticals including 5-ARI and/or alfa-blockers (reviewed by Tacklind et al. 2010). In cases of severe obstruction, hyperplastic nodules are resected transurethrally (TURP), and the diagnosis can be confirmed histologically. However, no correlations have been identified between histopathological parameters from needle biopsies and prostatic volume, or urinary obstructive symptoms (Viglione et al. 2002). Therefore, BPH cannot reliably be diagnosed using prostatic needle biopsies (Viglione et al. 2002).

## 1.4 Benign mimickers of prostate cancer

The most common benign mimickers of cancer are atrophy and foci of crowded glands with atypical features or atypical adenomatous hyperplasia (AAH, or adenosis) (Bostwick and Chang 1999). Other commonly known diagnostic pitfalls include; verumontanum hyperplasia, clear cell cribriform hyperplasia, nephrogenic adenoma and hyperplasia of the mesonephric ducts (Young 1988, Molinie et al. 2004). Basal cell hyperplasia (BCH) and atypical BCH can resemble both high grade prostatic intraepithelial neoplasia (HGPIN) and adenocarcinoma (Epstein and Armas 1992). Most of the lesions are readily distinguished from adenocarcinoma by the presence of basal cells and/or negative immunostaining for alpha-methylacyl-

CoA racemase (AMACR). Atypical BCH shows a multilayer staining pattern for the basal cell markers p63 and CK34betaE12 (Figure 2.) (Rioux-Leclercq and Epstein 2002). Because in cases of partial atrophy even the immunostaining pattern can resemble carcinoma, the differential diagnosis is based primarily on the glandular architecture of the H&E-staining (Herawi et al. 2005).

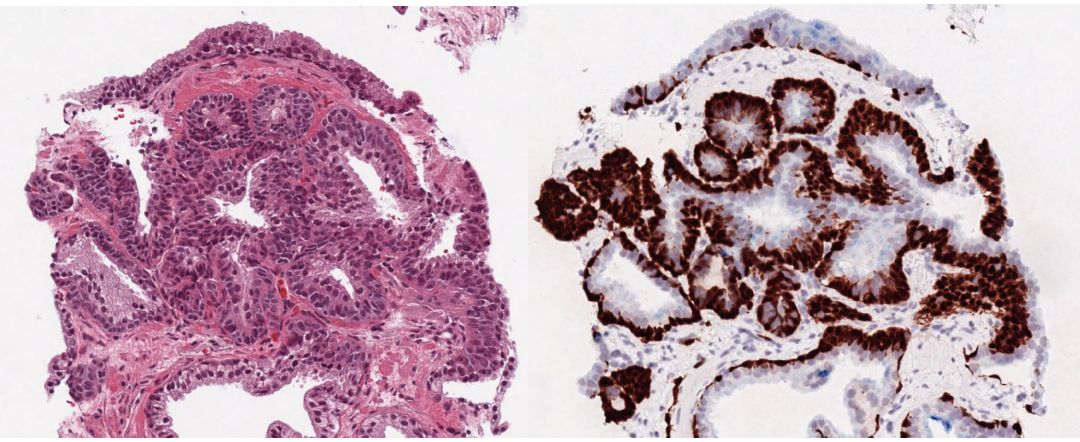

Figure 2. A focus containing atypical BCH. Left: nuclear atypia, hyperchromasia and stratification, H&E staining (100 x). Right: dual-color immunostaining shows intense brown staining of the basal cell layer with p63 and CK34betaE12 while luminal epithelial cells stain negative for AMACR (100 x).

## 1.5 Premalignant and suspicious lesions

### 1.5.1 High-grade prostatic intraepithelial neoplasia (HGPIN)

Historically, prostatic intraepithelial neoplasia was divided into three grades based on their morphological features (Sakr et al. 2004). Due to the problems associated with low reproducibility, this approach has been abandoned. Currently, the WHO recognizes two entities, low-grade and high-grade prostatic intraepithelial neoplasia (LGPIN and HGPIN), but only the latter has been shown to be associated with cancer (Qian et al 1997, Bostwick et al.1999, Sakr et al 2004). High-grade prostatic intraepithelial neoplasia is defined by the WHO as a neoplastic transformation of the epithelium lining the prostatic ducts and acini (Sakr et al 2004). Cytopathological features of HGPIN include a nearly uniformly increased

nuclear/cytoplasm-ratio, prominent nucleoli, and coarse chromatin. Typically, HGPIN presents multiple architectural patterns in combinations: tufted (97%), micropapillary (66%), flat (21%) or cribriform (19%) (Qian et al 1997). In addition, there are four variants recognized by the WHO: foamy, mucinous, signet-ring cell, and inverted HGPIN (Sakr et al 2004). In LGPIN, the diagnostic features are similar to HGPIN, but more inconspicuous and unevenly distributed (Sakr et al 2004).

HGPIN has morphological analogues in various organs, such as high grade dysplasia or carcinoma in situ of the colon or breast. It also shares molecular and genetic changes with adenocarcinoma (Qian et al. 1995, Meeker et al. 2002).

Recent studies of prostate needle biopsies have not shown an elevated risk of cancer in repeated biopsies for HGPIN, although the spatial association of HGPIN and adenocarcinoma in prostatectomy specimens is well-known (Postma et al. 2004, Laurila et al. 2009). It is believed that the significance of HGPIN in predicting cancer has diminished because of the sextant biopsy protocol, through which the majority of the cancers are detected (Herawi 2006). A recent Canadian study indicates, however, a strong association between HGPIN and subsequent adenocarcinoma on repeated biopsies. This study also reported a linear relationship between the extent/multifocality of HGPIN and cancer risk (Srigley et al. 2010). Recent molecular evidence also demonstrates that TMPRSS2:ERG gene fusion-positive HGPIN is associated with the same fusion status in matched cancer, a finding that could be used as a criterion for clinically significant HGPIN (Mosquera et al. 2008). However, the prognostic significance of TMPRSS2:ERG gene fusion in adenocarcinoma is yet to be determined (Attard et al. 2006, Saramäki et al. 2008).

Overdiagnosis of HGPIN is not uncommon (Bostwick and Ma 2007). Benign lesions in the differential diagnosis of HGPIN are atypical BCH (Figure 2) and benign central zone glands (Epstein and Armas 1992, Rioux-Leclercq and Epstein 2002, Bostwick and Ma 2007). The most important malignant lesions in the differential diagnosis of HGPIN are intraductal carcinoma and ductal carcinoma, which may be difficult to distinguish based on morphological criteria alone (Pickup and Van Der Kwast 2007). An example of HGPIN is shown in chapter 1.6 (Figure 4).

### 1.5.2 Proliferative inflammatory atrophy (PIA)

Proliferative inflammatory atrophy (PIA) has been linked to prostate carcinogenesis via HGPIN or via direct progression to cancer (De Marzo et al. 1999, De Marzo et al. 2003, Wang 2009). Epigenetic alterations, such as hypermethylation of cytidine-guanidine (CpG)-islands of the glutathione S-transferase P1 (GSTP1) gene promoter, have been noted in PIA lesions. These alterations may lead to cumulative genetic damage due to oxidative stress (De Marzo et al. 2003). The current understanding of PIA suggests that it is a benign lesion with a certain degree of genetic instability and that it can degenerate into prostate intraepithelial neoplasia or carcinoma (Woenckhaus and Fenic 2008). However, the clinical impact of PIA has not been confirmed, and it is not a routinely diagnosed entity in pathology reports. An example shown in Figure 3.

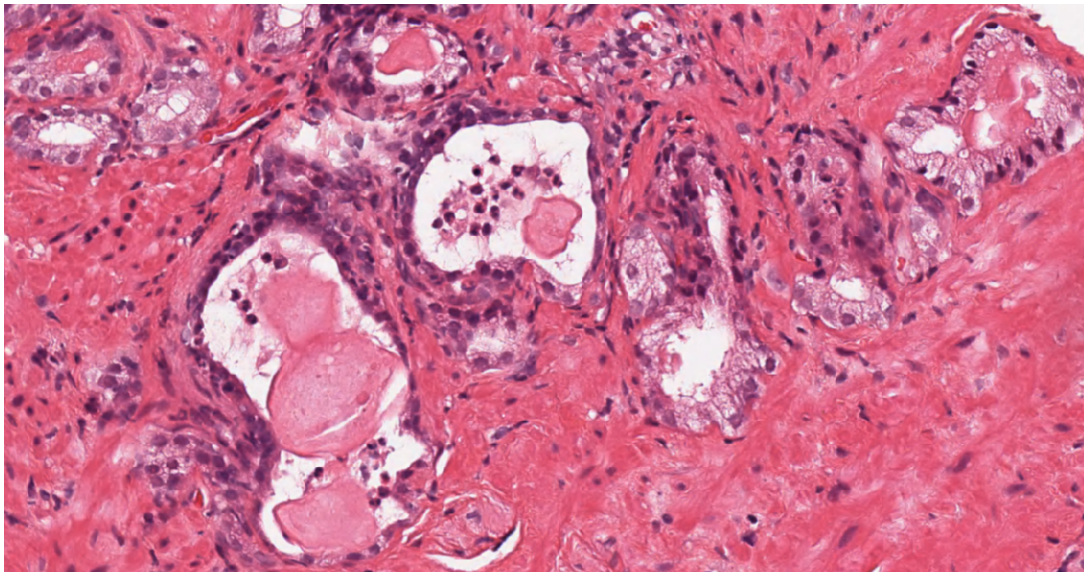

Figure 3. A focus of proliferative inflammatory atrophy (PIA) showing a few intraluminal neutrophils and regenerative epithelium of atrophic glands, H&E staining (200 x).

### 1.5.3 Atypical small acinar proliferation (ASAP)

Atypical small acinar proliferation (ASAP) does not exist as a discrete morphological entity but is a valid diagnosis (Iczkowski et al. 1999). The diagnosis of ASAP/suspicious for cancer is used when there are crowded glands with malignant features, but the focus is either too small to be considered cancer or does not fulfill the three major criteria of cancer (see 1.6 Adenocarcinoma). Most ASAP

diagnoses can be resolved to either benign or cancer with the aid of immunohistochemistry (Ng et al. 2007). Uniform requirements for diagnosing adenocarcinoma are lacking (e.g., the minimum number of glands). Before AMACR was introduced in the beginning of the 21st century, there were no positive markers for cancer, and the diagnosis of adenocarcinoma was based primarily on H&E staining. During that era, the median number of glands in foci diagnosed with ASAP was 11 and in cancerous foci was 17 (Bostwick and Iczkowski 1997). Currently, the number of glands needed for a cancer diagnosis by a uropathologist varies among between 2 and 10 (Egevad et al. 2006, Humphrey 2007). Other authorities recommend that no universal lower limit of glands can be assessed and that pathologists may diagnose cancer whenever they feel comfortable with the diagnosis (Van Der Kwast and Epstein 2010).

## 1.6 Adenocarcinoma

Approximately 80% of prostate cancers arise in the peripheral zone, 15% in the periurethrally located transitional zone, and 5% in the central zone of prostate (Cheng et al. 2005, Cohen et al. 2008). The observed frequencies roughly correlate with the different zone volumes (ml).

There are three uniformly accepted major criteria for prostate cancer diagnosis:

- Architectural disturbance: infiltrative small glands or cribriform glands too large or irregular to represent HGPIN
- Single cell layer (absence of basal cells)
- Nuclear atypia: nuclear and nucleolar enlargement (Iczkowsky et al. 1999, Humphrey 2007)

The same major criteria were recognized by Totten and his colleagues in the 1950`s (Totten et al. 1953).

Additional minor criteria may be used as helpful hints toward an adenocarcinoma diagnosis. These include (Algaba et al. 1996):

- Intraluminal wispy blue mucin (blue-tinged mucinous secretions)
- Pink amorphous secretions
- Mitotic figures
- Intraluminal crystalloids (see Figure 4)
- Adjacent HGPIN
- Amphophilic cytoplasm
- Nuclear hyperchromasia

In a survey of genitourinary pathologists by Egevad et al. (2006), four features were considered pathognomonic for prostate cancer. These were reported as follows by the participants: glomeruloid bodies (58%) (Figure 5), collagenous micronodules (also termed mucinous fibroplasias) (64%), circumferential perineural invasion (84%), and growth in fat (36%).

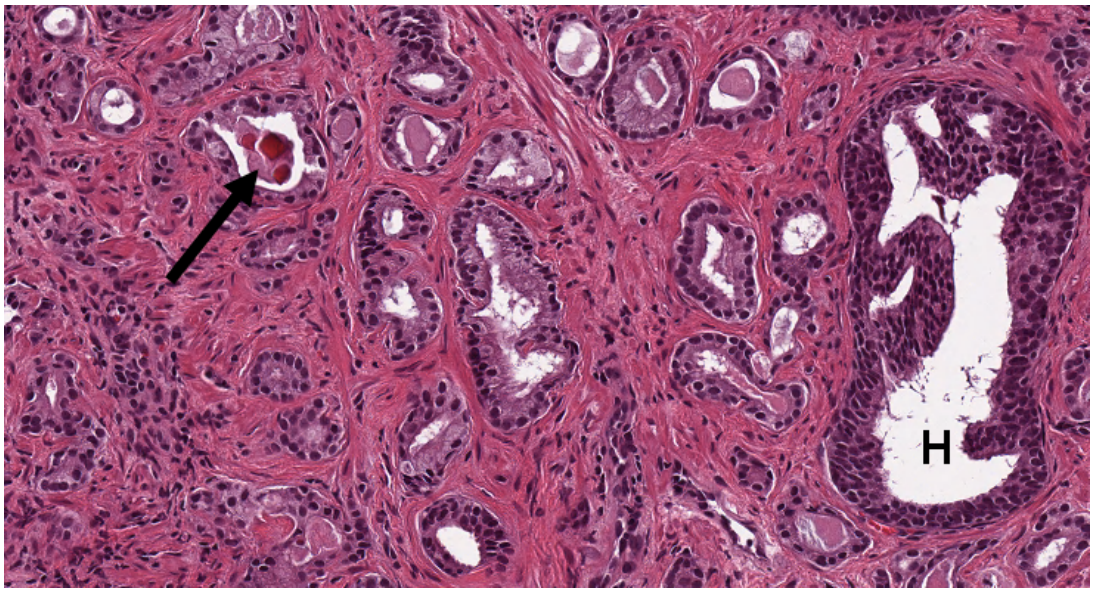


Figure 4. Small glands represent well-differentiated adenocarcinoma with a Gleason score 3+3=6. Eosinophilic amorphous secretions and cristalloids (arrow) can be noted inside the lumina of the glands. An adjacent HGPIN (H) shows an increased nucleus/cytoplasm ratio, hyperchromatic nuclei, amphophilic cytoplasm, and (cribriform) stratification of the epithelium. Basal cells are evident. H&E staining (200 x).

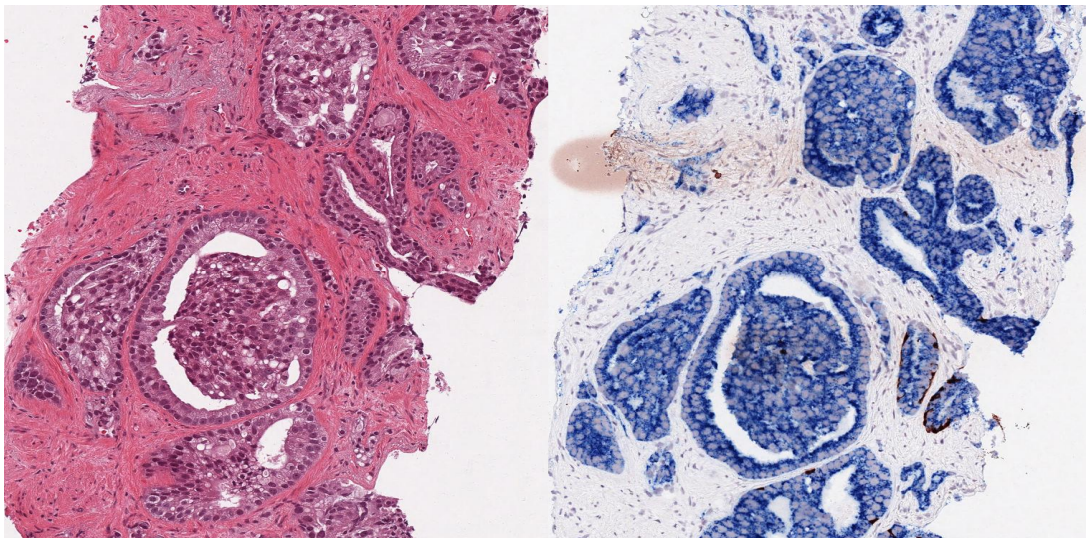

Figure 5. Glomeruloid structures are considered pathognomonic for prostate cancer. Left: H&E staining. Right: dual-color immunostaining (2IHC) showing strong positivity for AMACR and negative staining for basal cells (100 x).

# 2. Prostate cancer diagnostics in the PSA era

## 2.1 The impact of prostate specific antigen

Before PSA testing was introduced in late 1980s by Stamey and colleagues (1987), cancers were diagnosed by clinical symptoms, and needle biopsies were generally directed against a palpable mass (Mikuz 1997). Although PSA testing is now widely used in daily health care, the rationality of population-based PSA screening is still under debate (Barry 2009). Beginning five years after the inception of the PSA test, mortality due to prostate cancer has declined 4% annually in the U.S. (Ries et al. 2008). In the European Randomized Study of Screening for Prostate Cancer, PSA screening of 50-74-year-old men was associated with a significant decline in mortality. This achievement, however, was at the cost of a remarkable risk of overtreatment (Schröder et al.2009). At the same time, Andriole (2009) found no differences in prostate cancer-related mortality between PSA-screened and control patients during a 7-10-year follow-up (Andriole et al. 2009). In the U.S., most men over 50 years of age have been tested for serum PSA values (Ross et al. 2008). Moreover, 95% of urologists of the same age have had their PSA tested (Chan et al. 2006).

Cancers detected by screening are smaller and better differentiated than symptomatic cancers (Laurila et al. 2009). The more PSA tests are taken, the earlier cancers are detected, and the less cancerous tissue is observed in needle biopsies. Small, atypical foci are common, and a definitive cancer diagnosis is often difficult, resulting in considerable interobserver variability in the diagnosis of small, atypical lesions (Van der Kwast et al. 2010). Therefore, auxiliary immunohistochemical diagnostic tools have been developed.

## 2.2 Immunohistochemistry

### 2.2.1 Basal cell markers

Adenocarcinoma can be differentiated from benign glands by the absence of basal cells. However, it is not always a simple matter to distinguish basal cells in H&E-staining. The first markers aiding in the differential diagnosis of prostate cancer were basal cell layer markers, including MA-903, CK-903, and CK-HMW/CK34betaE12 (O´Malley et al.1990, Shah et al.1991). Various basal cell markers have been in use since immunohistochemical stainings were introduced into the clinical routine. The most commonly analyzed basal cytokeratins are CK34betaE12 and CK5/6 (Berney et al. 2005). Alternatively, the nuclear marker p63 may be used. Both CK34betaE12 and p63 are highly specific for basal cells (effectively 100%), with p63 demonstrating a slightly better sensitivity (Shah et al. 2002, Garcia et al. 2007). Because the basal cell layer is interrupted in the lesions most critical to differential diagnosis (HGPIN and AAH), basal cells are best visualized by co-staining for p63 and CK34betaE12 (Zhou et al. 2003, Jiang et al. 2005, Boran et al. 2010). In addition, co-staining is useful due to zonal variations in the immunophenotype of basal cells in the peripheral and transitional zones (Garcia et al. 2007).

### 2.2.2 Alpha-methylacyl-CoA racemase (AMACR, P504S, racemase)

Alpha-methyl-CoA racemace (AMACR) is a positive marker for prostatic neoplasias and was discovered using a high-throughput genetic analysis (Xu et al. 2000, Jiang et al. 2001). Since its identification, numerous studies have confirmed that it is specifically expressed in several dysplasias of other organs, such as Barret's esophagus and dysplasias associated with inflammatory bowel disease (Dorer and Odze 2006). In addition, AMACR is expressed in various malignancies, including adenocarcinoma of the esophagus and gastric and colon cancers (Dorer and Odze 2006, Lin et al. 2007, Truong et al. 2008). AMACR has high specificity

and sensitivity in the detection of HGPIN and prostatic adenocarcinoma (Jiang et al. 2001, Browne et al. 2004, Magi-Galluzzi et al. 2003), although there are variations between laboratories (Magi-Galluzzi et al. 2003). Since the introduction of AMACR approximately ten years ago, there have been several studies showing its usefulness in identifying prostate cancer and HGPIN (Browne et al. 2004, Carswell et al. 2006). AMACR expression is modulated by hormonal therapy, which is an important diagnostic pitfall to keep in mind when assessing follow-up biopsies after treatment (Sung et al. 2007).

### 2.2.3 Dual-color immunostains

The basic technique of dual-color horseradish peroxidase and alkaline-phosphatase immunostaining has been available for at least three decades (Valnes and Brandtzaeg 1984). Advances in the staining protocol and widespread use of immunostaining have now permitted their routine use. The antibodies in a given cocktail can be visualized with different commercially available chromogens, resulting in double-chromogen, or dual-color, stains (e.g., HistoBlue, Multivision kit; Vector Blue, Vector Laboratories, Inc., Burlingame, CA, USA; LPRed, Dako Denmark A/S, Glostrup, Denmark; Vector Red, Vector Laboratories, Inc., Burlingame, CA, USA). The specific combination of dual-color staining depends on the laboratory equipment. In dual-color stains, the diaminobenzidine (DAB)-horseradish peroxidase reaction is commonly used to stain the basal cell compartment brown. Alkaline-phosphatase activity assays using blue or red chromogens (e.g., Vector Blue and Vector Red, Vector Laboratories, Inc., Burlingame, CA, USA) are used to stain neoplastic epithelium. A cocktail of two antibodies for basal cells (e.g., p63 and CK-HMW, both DAB-brown) and one for neoplastic epithelium (AMACR, blue) enables three-antibody, two-chromogen staining.

Dual-color immunostainings are especially useful in the evaluation of prostate biopsies containing small foci suspicious for carcinoma or ASAP (Molinie et al.2004, Jiang et al.2005, Trpkov et al.2009). The major advantages are that both basal cells and neoplastic epithelium are visualized simultaneously on the same slide

with different colors (Figure 12 C and D), and the limited biopsy tissue available in the paraffin blocks is not exhausted as rapidly as with two separate immunostained slides (Hameed and Humphrey 2009).

# 3. Grading of prostate cancer

## 3.1 Gleason score - historical aspect

At least forty grading systems have been suggested for prostate adenocarcinoma (Humphrey PA 2003). One of the oldest and most used is the Gleason grading system, first published in the late 1960s (Gleason 1966, Mellinger et al. 1967). The World Health Organization (WHO)-Mostofi grading system has been widely used together with the Gleason system. In the original Mostofi grading system, both the architectural pattern (glandular differentiation) and nuclear grade (anaplasia) are combined to form three grades (Mostofi 1975). Later, a suggested modification by Dr. Mostofi´s group incorporated the count of mitotic figures to form five prognostic classes (Schroeder 1985). Although a high nuclear grade (gr. III) is associated with a high Gleason grade, the nuclear grade is sensitive to tissue fixation artifacts (Zhou et al. 2001). Therefore, determination of the nuclear grade is now avoided in many centers.

Several modifications to the original Gleason system have been suggested by Dr. Gleason (1974) himself, McNeal et al. (1986), and others. Despite gradual, minor adaptations, the Gleason score (GS) has stood the test of time as one of the most powerful prognostic factors for prostate cancer. As a system, the GS is an exception amongst grading systems of human cancers. While other grading systems are based on three or four grades, in the GS system the predominant and second most common cancer patterns are graded from 1 to 5 and summed to form a GS between 2 and 10 (Gleason 1966). The original patterns depicted by Dr. Gleason are shown in Figure 6.

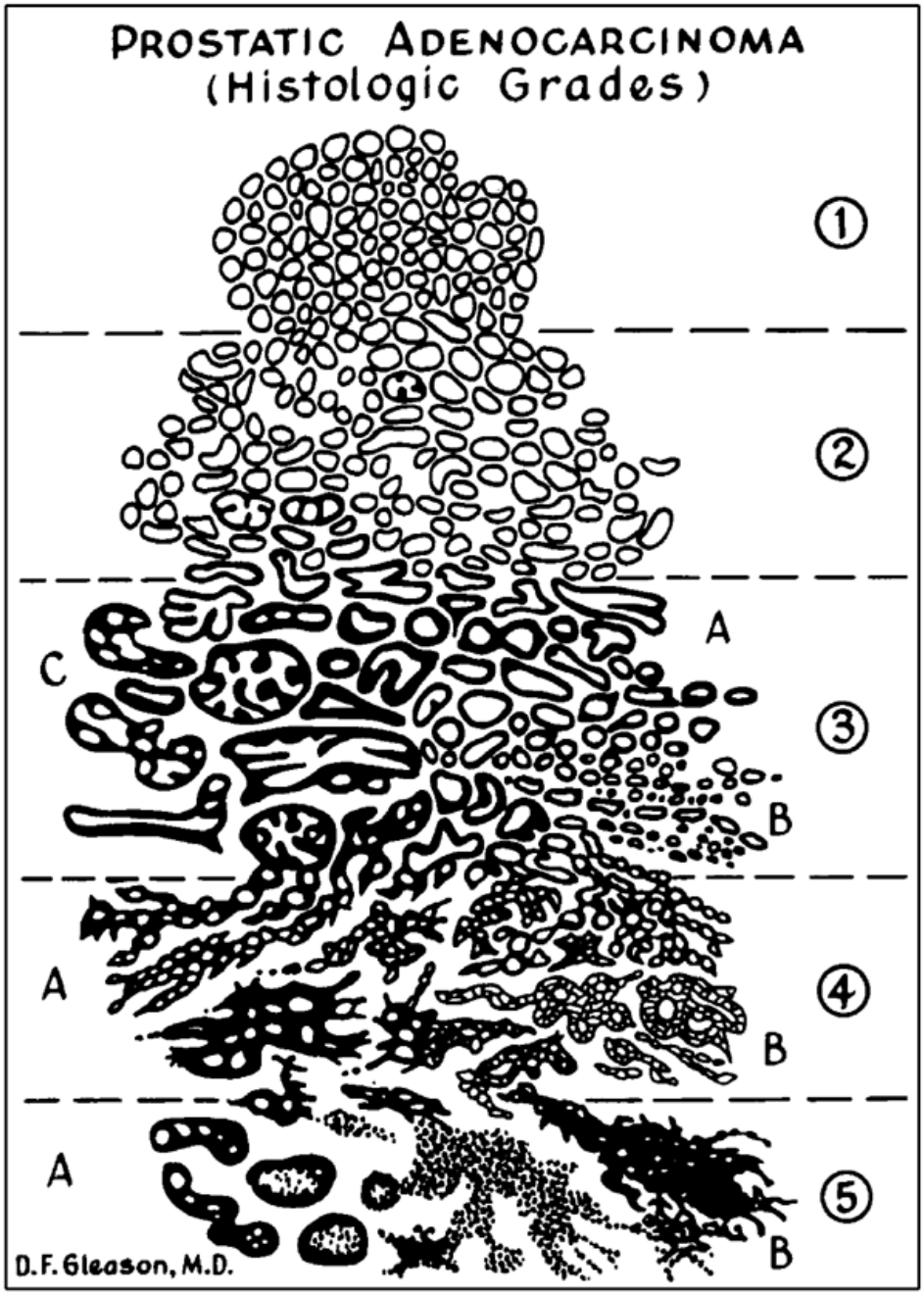


Figure 6. The classical Gleason patterns showing gradually increasing levels of dedifferentiation from 1 to 5. Reprinted by permission of Macmillan Publishers Ltd:[Modern Pathology](Humphrey 2004), copyright (2004).

## 3.2 Contemporary Gleason score - what is new?

In 2005, the International Society of Urological Pathology (ISUP) organized a consensus conference that yielded the first major revision of the original Gleason scoring system. Previously, GSs were reported to suffer from low reproducibility

and high interobserver variability among pathologists, although uropathologists performed slightly better (Alsbrook et al. 2001a, Alsbrook et al. 2001b). There was also need to update the system because of Gleason grade inflation and because upgrading of the GS following a radical prostatectomy was common. Several modifications were suggested and adapted worldwide, resulting in a "modified GS". These modifications have resulted in improved reproducibility, better correlations between the GS of the biopsy and the radical specimen, and a likely shift in the prognostic cut-off between low-grade and high-grade cancers from GS 6 vs. 7 to GS 3+4 vs. 4+3 (Helpap and Egevad 2006, Billis et al. 2008, Fine and Epstein 2008, Helpap and Egevad 2008, Fanning et al. 2009, Helpap and Egevad 2009).

In the original drawing of Gleason grades (Figure 6), there is a significant overlap between various grades. Cribriform structures are noted in all grades except Gleason grade 1, and gland fusion is noted on both sides of the dashed line between grades 3 and 4. Humphrey (2004) suggested that the original Gleason grade 1 may represent AAH and that before a diagnosis of GS 1+1=2 cancer is given the presence of any basal cells must be excluded. The 2005 ISUP uropathologist consensus statement stated that a GS 1+1=2 should not be used for any specimen type (Epstein et al. 2005). Additionally, it is likely that the original cribriform Gleason grade 3 patterns of carcinoma would currently receive a diagnosis of cribriform HGPIN if immunostains were performed (Amin 1994). A true fusion of the glands is always regarded as grade 4 according to the modified Gleason score (Epstein et al. 2005). In the ISUP Consensus Conference, cribriform structures are accepted as Gleason grade 3 provided that they have a round contour and are size of normal glands (Epstein et al. 2005). However, in a subsequent study representing only cribriform cancerous glands of putative Gleason grade 3 cases as defined above, most expert uropathologists graded all cribriform glands as Gleason grade 4 (Latour et al. 2008). Currently, it is recommended that all cribriform glands are graded Gleason grade 4 (Epstein JI 2010). Another evident change when comparing the old drawing (Figure 6) with the contemporary version (Figure 7) is that small, separate glands without lumina are considered as poorly differentiated and given a Gleason grade 4 (Figure 7). Infiltrating single cancer cells and strands of cancer cells, analogous to "indian files" in lobular carcinoma of the breast, are clearly depicted as Gleason grade 5. A solid pattern with or without comedonecrosis is another pattern accepted as Gleason grade 5.

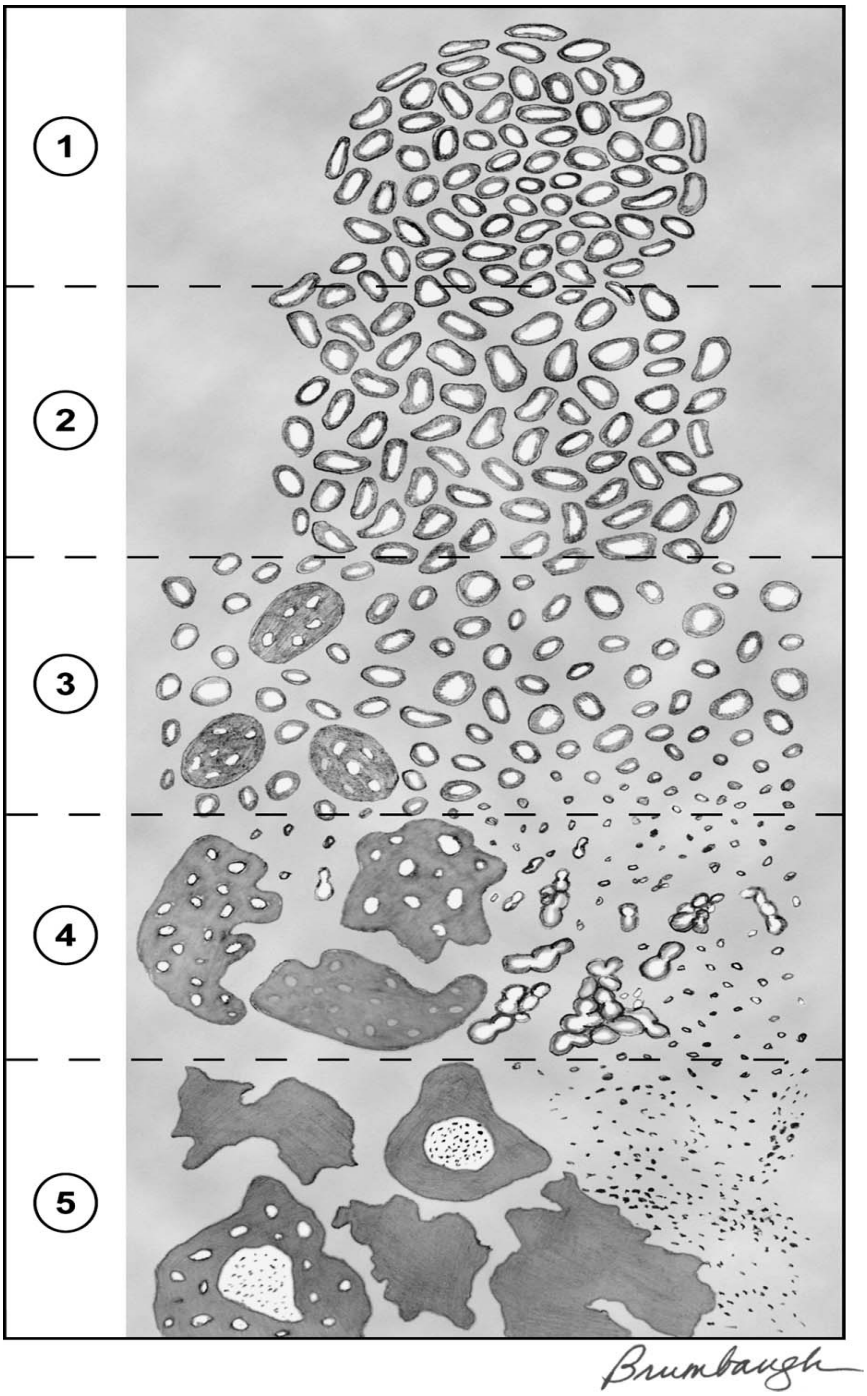


Figure 7. Refined Gleason patterns according to the modified Gleason scoring system. In general, the picture is more consistent with current recommendations than the original picture by Dr. Gleason (Figure. 6). However, some details are already discrepant with current recommendations. For instance, all cribriform structures should be under grade 4, with the exception of comedonecrosis. There is also some overlap of the smallest glands with minimal lumens from grade 3 up to grade 5. Reprinted with permission of Wolters Kluwer Health (from Epstein et al. 2005).

## 3.3 Grading of morphological variants of prostatic adenocarcinoma

### 3.3.1 Atrophic pattern adenocarcinoma

The atrophic variant of prostatic adenocarcinoma morphologically resembles atrophic benign glands with open lumens and a single-layered, flattened epithelium. However, cytologic atypia with enlarged nuclei and macronucleoli are noted at higher magnifications (Srigley 2004). This variant can be graded based on architecture using the Gleason pattern technique (Humphrey 2004).

### 3.3.2 Pseudohyperplastic adenocarcinoma

This variant is characterized by dilated glandular structures with a single layer of epithelial cells. Cells may show abundant cytoplasm similar to hyperplastic cells. Lack of the basal cells is easily demonstrated by immunohistochemistry (IHC) (Figure 8). The pseudohyperplastic variant is graded as Gleason grade 3 by most uropathologists of the ISUP consensus panel (Epstein et al. 2005).

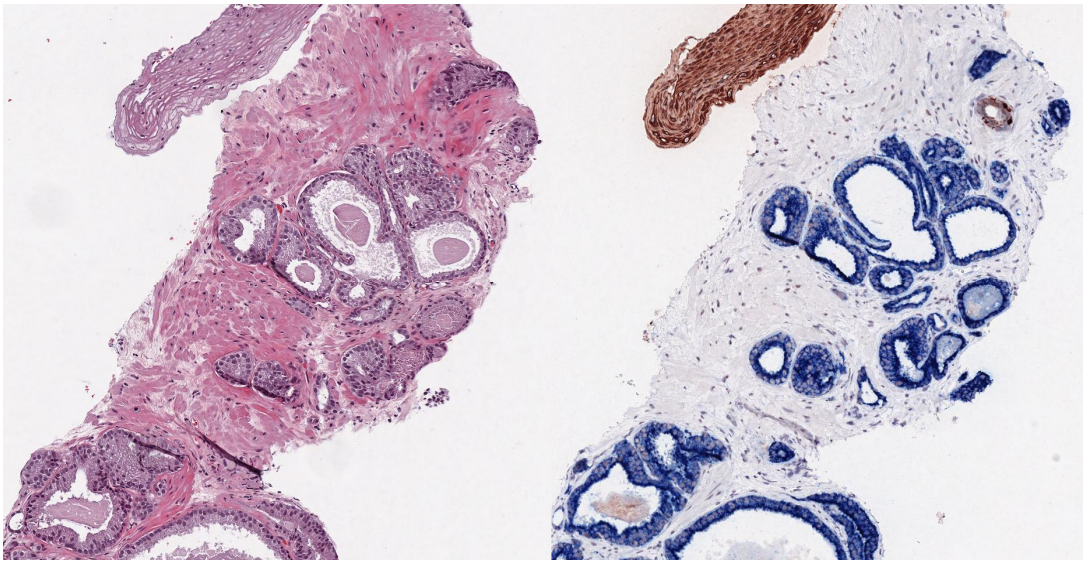

Figure 8. Left: benign-appearing dilated glands with a single-layered luminal epithelium, H&E staining. Right: dual-color staining reveals a lack of basal cells and uniform positivity for AMACR. Squamous epithelium noted on the top of the right-hand picture serves as an internal control for the basal cytokeratin stain (100 x).

### 3.3.3 Foamy gland carcinoma

Foamy gland carcinoma was characterized by Nelson and Epstein (1996) as consisting of glands with cytologically benign-appearing cells with abundant xanthomatous cytoplasm (Figure 9). In most cases, cells lack prominent nucleoli, which is one of the three major criteria used to diagnose prostate cancer (Nelson and Epstein 1996). The foamy appearance of the tumor cells results from numerous alcian blue-positive intracytoplasmic vesicles (Tran et al. 2001). Glandular formation is usually consistent with Gleason pattern 3 (Epstein et al. 2005). However, some of these tumors may behave aggressively (Tran et al. 2001).

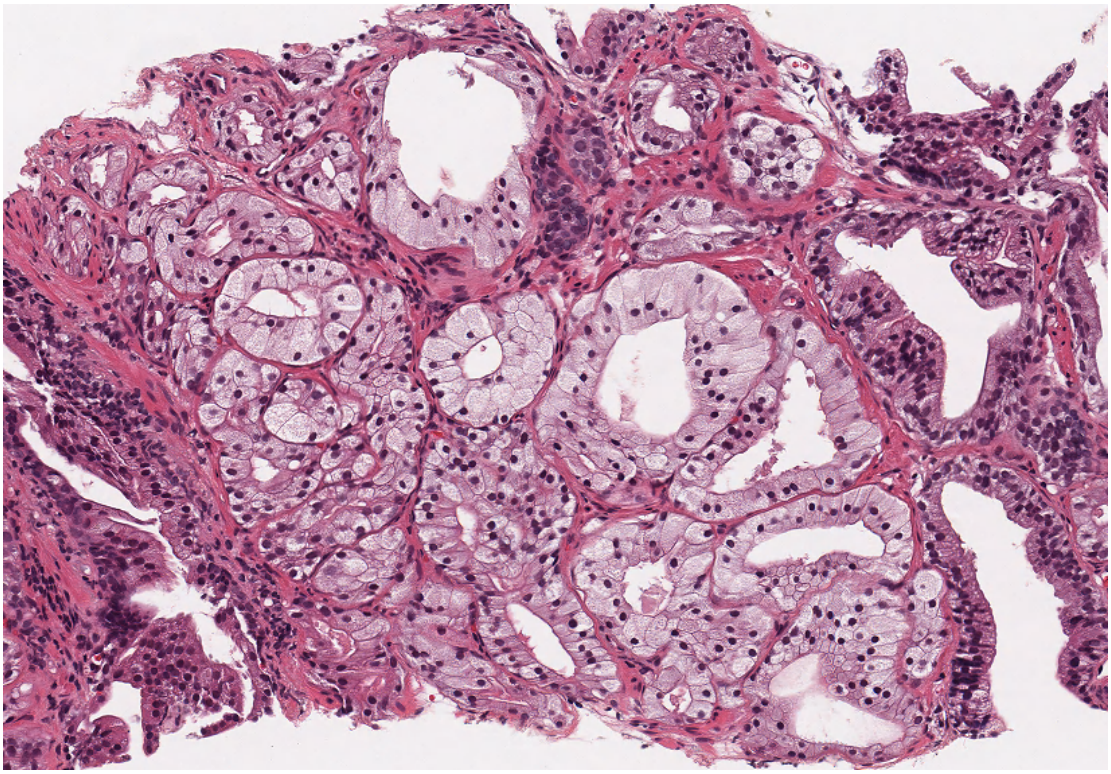

Figure 9. Foamy gland carcinoma. Cancer cells have xanthomatous-appearing cytoplasm and very small, benign-appearing round nuclei. H&E staining (200 x).

### 3.3.4 Colloid (mucinous) adenocarcinoma

Earlier, mucinous variants by definition were considered as Gleason grade 4 (Humphrey 2004). Most mucinous cancers are have a Gleason grade 4 pattern resembling cribriform irregular glands floating within a mucinous matrix, but the

glandular formation may occasionally be closer to that of Gleason grade 3 (Figure 10.) (Epstein et al. 2005). The current consensus of uropathologists is that mucinous carcinomas should be graded based on glandular formation, which is usually of grade 4 but sometimes of grade 3 (Epstein et al. 2005). The prognostic significance of this refinement is unknown.

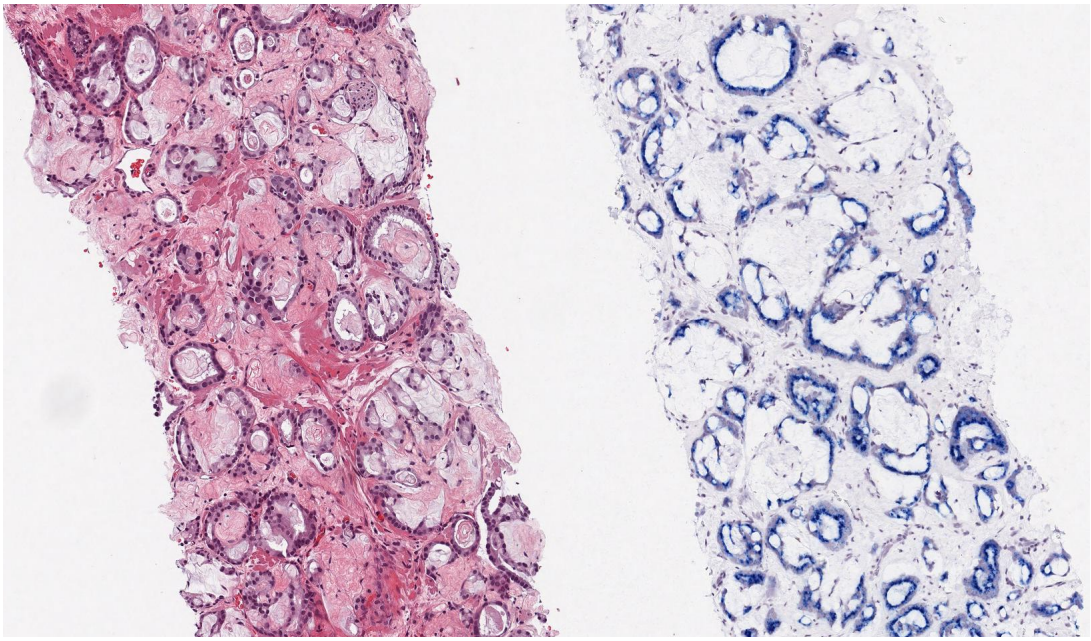

Figure 10. Mucinous adenocarcinoma showing extracellular mucin production. Mucin is predominantly intraglandular but also leaks to the stroma. Glandular formation is most easily observed on the right image (2IHC), where most of the glands are fused (Gleason pattern 4), but separate glands are also evident (Gleason pattern 3) (100 x).

### 3.3.5 Signet-ring cell carcinoma

This variant appears similar to signet-ring cell cancers in other locations. Glandular formation is absent, and the cells produce intracellular mucin, protruding flattened nuclei to the periphery. When these cells are present in significant amounts (>25%), the cancer is referred to signet-ring cell cancer. This variant represents the most aggressive Gleason grade of 5. According to the original studies by Dr. Gleason, the signet-ring cell variant is very rare (Humphrey 2004). Intracytoplasmic vacuoles, however, are common in prostatic adenocarcinoma and should be considered separately from mucin-containing signet-ring cells (Figure 11) (Epstein et al. 2005).

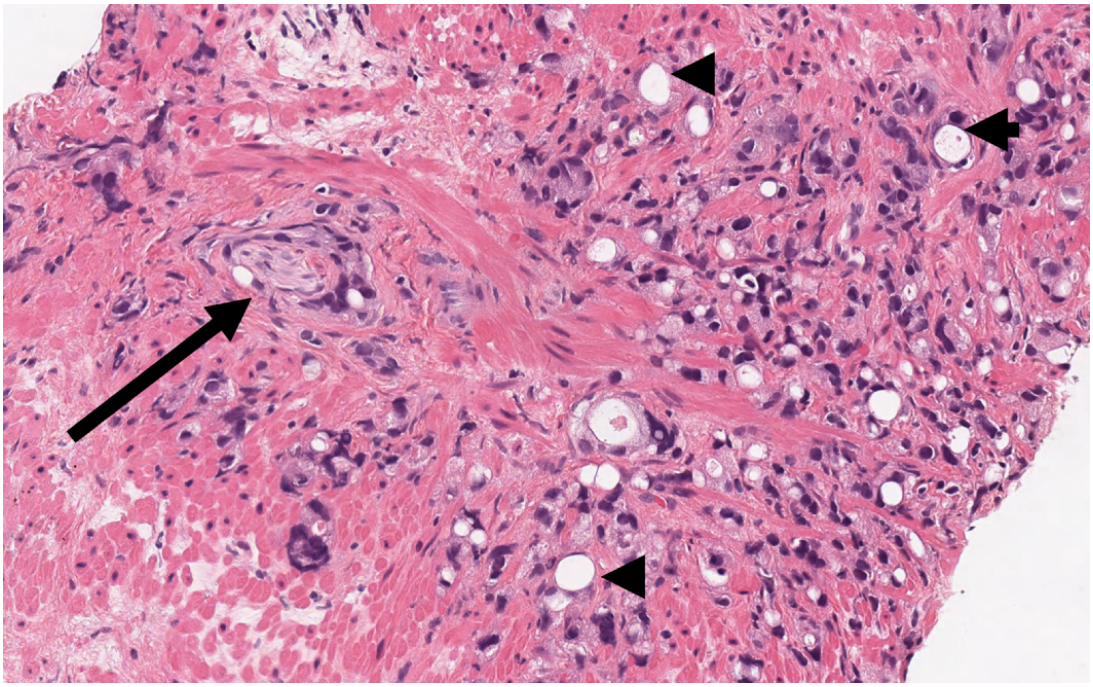

Figure 11. Poorly differentiated adenocarcinoma of a GS 5+5=10 cancer showing intracytoplasmic vacuoles resembling signet-ring cells (arrowheads). However, this is not considered a true signet-ring cell cancer. Perineural invasion is thought to be one of the few pathognomonic features for prostate cancer (arrow). H&E staining (200 x).

### 3.3.6 Other variants of acinar adenocarcinoma

In the clear cell/hypernephromatoid variant, cells have abundant cytoplasm and small nuclei. Tightly packed or fused glandular structures with a small lumen may form. Although this pattern resembles well-differentiated clear cell renal cell carcinoma of Fuhrman grade 1, it behaves aggressively and actually forms the original Gleason grade 4 cancer (Gleason et al.1966).

Other rare variants recognized by the WHO include the oncocytic, lymphoepithelioma-like, and sarcomatoid variants (carcinosarcoma) (Epstein et al. 2004).

### 3.3.7 Ductal adenocarcinoma and comedocarcinoma

Ductal adenocarcinoma is defined by the WHO as a “subtype of adenocarcinoma composed of large glands lined by tall pseudostratified columnar cells” (Yang et al

2004). Intraductal papillary fronds or cribriform structures are considered characteristic (Brinker et al. 1999). The three subtypes of ductal adenocarcinoma recognized by the WHO are the papillary, cribriform, and solid types (Yang et al. 2004). Glandular elements resembling endometrial carcinoma may also be noted, although use of term “endometrioid carcinoma” is discouraged by the WHO (Yang et al 2004). In ductal carcinoma, the cytoplasm is usually amphophilic, and basal cells are rarely noted (Yang et al 2004). The major differential diagnoses are with intraductal carcinoma and cribriform HGPIN. Ductal carcinomas are mostly equivalent to Gleason grade 4 as recommended by the ISUP 2005 consensus panel (Epstein et al. 2005) and the WHO (Yang et al. 2004). Ductal carcinoma with central comedonecrosis is graded as Gleason grade 5 (Humphrey 2004, Yang et al. 2004).

Ductal carcinoma is still a debated concept. Although diagnostic criteria have been established, there is no molecular or even morphological evidence that ductal carcinoma is a separate entity (Pickup and Van Der Kwast 2007). The largest published series, consisting of 371 cases of ductal adenocarcinoma, showed an association with increased disease-specific mortality risk and significantly lower PSA values when compared to normal acinar-type adenocarcinoma (Morgan et al. 2010). These 371 ductal carcinomas were identified from a register containing 443 251 prostate cancer patients, with a reported incidence of only 1:1195 cancers (Morgan et al. 2010). In another study by Bock and Bostwick (1999), 5% of peripheral acinar cancers showed features traditionally associated with ductal cancer, namely papillary and cribriform growth patterns and a lack of basal cells. Thus, ductal features are more common in regular peripheral adenocarcinoma than in presumed central cancers of ductal origin. Moreover, the nomenclature is ambiguous and warrants classification under conventional types of adenocarcinomas (Pickup and Van Der Kwast 2007). In conclusion, the current expert opinion is that if “pure” ductal carcinoma of the prostate exists, it is rare.

## 3.4 Reporting prostate needle biopsies

In 2005, the Gleason grading system of needle biopsies experienced its first major revision (Epstein et al. 2005). The so-called modified Gleason grading system is based on the opinions of the consensus panel of the world's leading uropathologists at the 2005 ISUP Consensus Conference. The most important new recommendations of the conference were:

- The most aggressive Gleason pattern should always be incorporated as a part of the GS, regardless of its area (even if <5%)
- A Gleason score of 1+1=2 should not be used (in any type of specimen)
- A small area (<5%) of less aggressive cancer should be ignored

Prior to the ISUP Consensus Conference, Epstein (2000) suggested that a diagnosis of GS 2-4 adenocarcinoma could be assessed "rarely if ever" based on needle biopsies. This statement was based on several observations. First, a large amount of radical prostatectomy samples at Johns Hopkins showed that all cancers diagnosed with GS 2-4 on needle biopsies were upgraded on subsequent radical prostatectomies. Second, because GS 2-4 cancers arise in the transitional zone, they are not usually biopsied. Third, GS 2-4 cancers are round nodules by definition; it is impossible to see the edges of an entire nodule in a biopsy core.

All of the aforementioned recommendations have now been generally accepted and show an improved correlation with radical specimens (Helpap and Egevad 2006, Billis et al. 2008). However, it has been shown that older pathologists still diagnose GS 2-4 adenocarcinomas on needle biopsies more frequently than their younger colleagues (Egevad et al. 2005).

## 3.5 Current controversies

One of the most concerning issues regarding prostate needle biopsies today is the lack of standardized manufacturing. The practice varies between the USA and Europe and between countries and laboratories. In a recent, unpublished survey by the European Network of Uropathologists (ENUP), slightly more than half of laboratories process prostate needle biopsies individually, while the rest use some form of pooled biopsy protocols (between 2 and 6 biopsies per block) (Lars Egevad 2011, personal communication). Because the processing method used affects reporting of prostate needle biopsies, there are direct clinical and prognostic implications. Clinicians and uropathologists should be aware that the Gleason system used impacts patient prognosis and treatment (Montironi et al. 2010). The ISUP recommends assessing the worst GS based on needle biopsies submitted in separate containers (Epstein et al. 2005). However, the ISUP panel did not reach a consensus for cases in which multiple cancer-containing cores are submitted in one specimen container (Epstein et al. 2005). Moreover, when multiple cores are positive, Europeans more frequently report an additional overall GS at the end of the pathology report (Egevad et al. 2005). In addition, although cancers are now identified at earlier stages, Gleason scores have been increasing for the last two decades in the absence of a true biological change (Albertsen et al. 2005a). Changing scoring systems together with variable processing and reporting methods are suboptimal contexts in which to ask clinicians to make the best treatment choices.

# 4. Prognostic factors in prostate cancer

## 4.1 Prostate-specific antigen (PSA)

The pre-treatment serum PSA value (ng/ml) is not only a diagnostic marker for prostate cancer but also one of the strongest prognostic factors for this disease (Partin 2001, Graff et al. 2007). The PSA value may be used in patient follow-up as a surrogate marker for disease progression (Stamey et al.1987, Strohmaier et al. 1999). If post-treatment PSA values remain low for a 5-year period, the probability of subsequently developing metastasis is approximately 1% (Stock et al. 2009). In addition, response in PSA value is an independent prognostic factor for patients treated with endocrine therapy (Palmberg et al. 1999). In rare instances, the PSA value cannot be reliably used as a prognostic marker for some variants of prostate cancer, such as small cell cancer and possibly “true” ductal carcinoma (Brinker 1999, Wang and Epstein 2008, Morgan et al. 2010).

## 4.2 Gleason score

Forty years since its inception, the Gleason score has remained the most widely used grading system because of its extremely strong prognostic value (Partin et al. 2001, Egevad et al. 2002, Stephenson et al. 2005, Graff et al. 2007, Waltz et al. 2009). The scores, however, have changed over time due to stage migration and grade inflation, the so-called Will Rogers phenomenon, which is well known in various cancers, including prostate cancer (Thompson et al. 2005). The predominant and the second most common Gleason patterns are no longer combined *per se*, due to the following adaptations to the original GS system:

- In the case of a radical prostatectomy specimen with separate tumor nodules, the grade of the dominant tumor nodules is recommended (Epstein et al. 2005)
- Because it is unlikely that, for instance, a GS 2+2=4 central nodule would change the poor prognosis associated with a peripheral, undifferentiated cancer, the GS needs to be assessed only from the aggressive nodule (Epstein et al. 2005).

There is also some evidence suggesting that the most aggressive Gleason grade today may be a stronger prognosticator than the original Gleason score (Vis et al. 2007).

## 4.3 Clinical tumor, nodes and metastasis (cTNM) – stage

The clinical tumor stage (cT-stage) has previously been an important prognosticator. However, frequent PSA testing has led to the detection of smaller cancers, which are unpalpable in a DRE. In addition, nearly one third of cT-stages are incorrectly assessed for other reasons (Reese et al. 2011). In conclusion, the cT-stage may lack independent prognostic power in the PSA era, most likely due to changes in the detectable volume of cancer by urologists and radiologists and because of better predictive parameters in core biopsies (Reese et al. 2011).

## 4.4 Prognostic findings from the radical prostatectomy specimen

### 4.4.1 Pathological tumor, nodes and metastasis (pTNM) -stage

Pathological tumor stage (pT-stage) is based on the evaluation of a radical prostatectomy specimen. The specimen should be completely embedded in a paraffin block, preferably on a macroblock, which are especially useful for assessing tumor volume, multifocality and marginal positivity. According to the TNM

Classification of Malignant Tumours – 7th Edition by the International Union Against Cancer (UICC), the following rules are applied in pathological staging of prostate cancer:

pTX. Primary tumor cannot be assessed

pT0. No evidence of primary tumor

pT2. Tumor confined within prostate

pT2a. Tumor involves one half of one lobe or less

pT2b. Tumor involves more than half of one lobe, but not both lobes

pT2c. Tumor involves both lobes

pT3. Tumor extends through the prostatic capsule[1]

pT3a. Extracapsular extension (unilateral or bilateral), including microscopic bladder neck involvement

pT3b. Tumor invades seminal vesicle(s)

pT4. Tumor is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall

[1] Invasion into the prostatic apex or into (but not beyond) the prostate capsule is not classified as pT3, but as pT2.

Based on large series, local cancers with <pT3 have a very favorable long-term prognosis in terms of cancer-specific mortality, comparable even to that of age-matched controls (Zincke et al. 1994). Seminal vesicle invasion (pT3b) predicts early PSA progression (Waltz et al. 2009).

If an iliac lymphadenectomy is performed pre-/intraoperatively, the pN stage can be assessed. Lymph node invasion (pN1) is predictive for early PSA recurrence following a radical prostatectomy (Waltz et al. 2009). The pathological (p)M category is seldom used because metastases are most often diagnosed clinically based on findings from bone scintigraphies and serum PSA values (Oesterling 1993, Montie 1995).

### 4.4.2 Surgical margin status

A positive surgical margin status is a known risk factor for earlier PSA recurrence (Blute et al 1997, Swindle et al 2005, Vis et al 2006, Marks et al. 2007). It may also predict clinical recurrence of the disease and the development of distant metastases (Pfitzenmaier et al. 2008). Results regarding the prognostic significance of specific site of margin positivity (Blute et al. 1997, Vis et al. 2006) or the linear extent of margin positivity (Emerson et al. 2005, Vis et al 2006, Marks et al. 2007) remain controversial. Multiple positive sites or bilaterally positive surgical margins may indicate an earlier biochemical recurrence (Emerson et al. 2005, Somford et al. 2011). Prognostic factors are closely associated with each other; it is not unexpected, therefore, that pre-treatment PSA values and Gleason scores are predictive of margin positivity (Somford et al. 2011).

### 4.4.3 Capsular status

The outer surface of the prostate consists of a layer of concentrically organized fibromuscular bands that are inseparable from the prostatic stroma. This fibromuscular band disappears at the apex (Ayala et al. 1989). Therefore, the prostate is considered to lack a true capsule. The fibromuscular band around the prostate, whether referred to as the capsule or pseudocapsule, is important to recognize because of its effects on pathological staging. Although it is somewhat controversial, the pathological stage pT3a is defined by the UICC as extracapsular extension (see above). Most pathologists would agree with a staging of pT3a when cancer infiltration is detected in the periprostatic fat but when less than half of the periprostatic surfaces harbor fat on the radical prostatectomy specimen, making the evaluation of extraprostatic extension unreliable (Hong et al. 2003). In needle biopsies, cancer infiltration into the fat is considered a reliable mark for stage pT3a, but intraprostatic fat may exist in up to 4% of prostates (Nazeer et al. 2009).

### 4.4.4 Prognostic groupings according to TNM stage, PSA value and Gleason score

The prognosticators of prostate cancer can be used to create nomograms. Three of the strongest prognostic factors have been used in combination to create five prognostic categories (TABLE I) (Sobin et al. 2009). Patients with extraprostatic extension (pT3a-b) and patients with nodal or distant metastasis belong to a high-risk category independent of PSA value or GS. However, most prostate cancers are multifocal and bilateral (pT2c) and belong to an intermediate risk category. Therefore, new strategies, including molecular biomarkers, have been developed to subdivide these patients into low- and high-risk groups.

TABLE I - Prognostic groupings according to TNM, PSA and GS

| | | | | | |
|---|---|---|---|---|---|
| I | | T1a – c | N0 | PSA < 10 | GS ≤ 6 |
| | | T2a | N0 | PSA < 10 | GS ≤ 6 |
| IIA | | T1 a – c | N0 | PSA < 20 | GS 7 |
| | | T1 a – c | N0 | PSA ≥ 10 < 20 | GS ≤ 6 |
| | | T2a,b | N0 | PSA < 20 | GS ≤ 7 |
| IIB | | T2c | N0 | Any PSA | Any GS |
| | | T 1-2 | N0 | PSA ≥ 20 | Any GS |
| | | T 1-2 | N0 | Any PSA | GS ≥ 8 |
| III | | T3a, b | N0 | Any PSA | Any GS |
| IV | | T4 | N0 | Any PSA | Any GS |
| | | Any T | N1 | Any PSA | Any GS |
| | M1 | Any T | Any N | Any PSA | Any GS |

Adapted from the 7th version of TNM classification of malignant tumours by UICC

## 4.5 Prognostic findings from needle biopsy specimen

In addition to the generally accepted prognostic factors for radical prostatectomy specimens, a large number of additional predictive and prognostic parameters are suggested for needle biopsies. Some of these factors are complex and are not used in

routine clinical work. Only the most important and clinically relevant prognostic factors are presented below.

### 4.5.1 Number and length of biopsies

The cancer detection rate depends on the total length of the biopsies (Iczkowski KA et al. 2002). Therefore, the length of the biopsies is one of the few prognostically significant parameters that can be influenced by education and by adapting special techniques (Bostwick et al. 2010). Increasing the number of the biopsy cores from 6 biopsies to 10 or more has increased (up to 35%) cancer detection rates (Eskew et al. 1997, Eskicorapci et al. 2005, Elabbady and Khedr 2006). Moreover, objective methods for reporting the quality of the biopsies have been developed (Mondet et al. 2009).

### 4.5.2 Total percentage of cancer (TPC)

The total percentage of cancer on the needle biopsies is predictive of a pT stage ≥3 in the radical prostatectomy specimen and of biochemical recurrence following radical prostatectomy (Villamon-Fort et al. 2007, Brimo et al. 2008, Rajab et al. 2010, Quintal et al 2011).

### 4.5.3 Greatest percentage of cancer (GPC)

The greatest percentage of cancer in a single biopsy core is associated with a pT stage ≥3 in the radical prostatectomy specimen (Brimo et al. 2008). This metric has also shown independent prognostic value in surgically treated patients (Nelson et al. 2002). In recent comparisons of various morphometric measurements of prostate needle biopsy tissue, however, GPC was the only parameter not associated with PSA recurrence (Quintal et al 2011).

### 4.5.4 Percentage of cancer-positive cores (CPC)

In the past, the use of systematic sextant biopsies for non-palpable tumors showed only a weak correlation between cancer-positive needle biopsy cores and findings from the prostatectomy specimen (Noguchi et al. 2001). More recently, both the number of cores positive for cancer and the percentage of cancer-positive cores have shown independent prognostic power (Winkler et al. 2004). In a study by San Francisco et al. (2004), the percentage of positive cores was a better predictor of cancer recurrence than the pre-treatment PSA value. Linear measurements of the cancer have been encouraged by other investigators (Haggarth et al. 2005). Recently, Rajab et al. (2010) has addressed this issue in a large study in which the percentage of cancer and the percentage of cancer-positive cores were independent prognostic factors, superior to the total length of the cancer (mm) and the number of cancer cores.

### 4.5.5 Perineural invasion (PNI)

Nerves are located near the capsular area of the prostate, and the most common spreading route for prostate cancer is capsular penetration into the perineural space (Villers et al. 1989). Perineural invasion (PNI) is a common event (approximately 75%) in radical prostatectomy specimens (Maru et al. 2001) and therefore lacks prognostic significance (Merrilees et al. 2008). However, PNI detected in core biopsies is associated with adverse findings in the radical prostatectomy specimen (Loeb et al. 2009, Moussa et al. 2009) and is a significant prognostic factor in univariate analyses of surgically treated patients (Nelson et al. 2002). The presence of PNI also correlates with earlier PSA recurrence in patients treated with external beam radiotherapy (Bonin et al.1997, Yu et al. 2007).

### 4.5.6 Worst (highest) Gleason score

In the traditional Gleason scoring system used for needle biopsy specimens, only the predominant and second most common cancer patterns are considered. According to the modified Gleason scoring system established by the ISUP 2005 Consensus

Conference, the most aggressive cancer observed in the needle biopsies should be incorporated as the second pattern even if only present in small amounts (Epstein et al. 2005). In the case of individually embedded biopsies, each biopsy is given a separate Gleason score. The primary and secondary Gleason grades from the biopsy with the highest GS are used by clinicians in pre-treatment nomograms (Memorial Sloan-Kettering Cancer Center at http://www.mskcc.org/applications/nomograms/prostate/PreTreatment.aspx 25.02.2011).

In a few studies, the worst Gleason score showed a better correlation than the overall Gleason score to findings from subsequent radical prostatectomy specimens (Kunju et al. 2009, Kunz and Epstein 2003, Poulos et al. 2005). Furthermore, the length (mm) of the most aggressive cancer focus is predictive for biochemical (PSA) recurrence (Brimo et al. 2008).

## 4.6 Prognostic molecular markers

There is an ongoing, vigorous search for new molecular and genetic markers for prostate cancer. The main pathways under investigation, including cell cycle control, androgen receptor signaling, genomic instability, adhesion molecules, death and apoptosis, signal transduction and angiogenesis, are extensively reviewed by Quinn et al. (2005) and Buhmeida et al. (2006). Some of the most studied single biomarkers with potential clinical relevance are presented in TABLE II. Recently, a promising genetic approach utilizing a tumor-derived RNA expression signature of multiple (n=31) cell cycle progression-related genes was developed (Cuzick et al. 2011). The authors concluded that the cell cycle progression signature is a robust prognostic marker that may have an essential role in treatment decisions following additional validation. The following chapters concentrate on the biomarkers used in studies I and III.

**TABLE II - prognostic biomarkers in prostate cancer**

| Molecule* | n:o of cases | analysis type | Effect on outcome | Reference |
|---|---|---|---|---|
| AZGP1 | 225 | multivariate | Strong immunostaining is associated with a decreased risk of recurrence | Lapointe et al. (2004) |
| Bcl-2 | 175, 1172 | uni-/multivariate | Increased expression is associated with bichemical failure and death from prostate cancer | Bauer et al. (1996), Concato et al. (2009) |
| E-cadherin | 56, 1220(TMA), 259 | univariate | Low/aberrant expression associated with high-grade tumors. | Ross et al. (1994), Rubin et al. (2001), Rhodes et al. (2003) |
| EZH2 | 249, 259 | univariate | Moderate to strong expression is prognostic. Increased expression in metastatic prostate cancer. | Varambally et al. (2002), Laitinen et al. (2008), Rhodes et al. (2003) |
| Hepsin | 78, 11, 259 | uni-/multivariate | Selectively upregulated in cancer; prognostic data is discordant. | Dhanasekaran et al (2001), Magee et al (2001), Rhodes et al (2003) |
| HIF-1 alpha | 201+289 | multivariate | Independent prognosticator of biochemical failure in patients treated with radiotherapy | Vergis et al. (2008) |
| Ki-67 | 190, 111, 808 | multivariate | Independently predicts survival and associates with Gleason score in most studies. | Aaltomaa et al. (1997), Bubendorf et al. (1998), Berney et al. (2009) |
| MCM7 | 223, 249 | uni-/multivariate | Increased expression and gene amplification associated with biochemical recurrence time | Ren et al (2006), Laitinen et al (2007) |
| MTA1 | 300 | multivariate | Overexpressed in CRPC. Decreased expression associated with PSA recurrence in cT1-2. | Hofer et al. (2004) |
| MUC1 | 225 | multivariate | Positive immunostaining is associated with an increased risk of recurrence | Lapointe et al. (2004) |
| Osteopontin | 289 | multivariate | Independent prognosticator of biochemical failure in patients treated surgically | Vergis et al. (2008) |
| p16 | ? | multivariate | Predictive for cancer-specific death in conservatively treated cT1-2 cancer. | Kudahetti et al. (2010) |
| p53 | 137, 109, 175, 1172 | uni-/multivariate | Mutations and loss of heterozygosity associated with long-term cancer-specific death. | Visakorpi et al (1992), Shurbaji et al. (1995), Bauer et al. (1996), Concato et al. (2009) |
| PIM1 | 78(?) | multivariate | Decreased expression is predictive for PSA recurrence. | Dhanasekaran et al (2001) |
| pTEN | 125, 308 | uni-/multivariate | pTEN deletion with TMPRSS2:ERG fusion predicts PSA recurrence and death from prostate cancer. | Yashimoto et al. (2008), Reid et al (2010) |
| Spink1 | 1197, 186 | multivariate | Increased expression associated with progression-free survival | Tomlins et al (2008), Leinonen et al (2010) |
| TMPRSS2:ERG | 445, 253, 178 | multivariate | Discordant results about predictive value. Duplication of gene fusion may be significant. | Attard et al. (2008), Saramäki et al (2008), Leinonen et al (2010) |
| VEGF | 201+289 | multivariate | Independent prognosticator of biochemical failure in patients treated with radiotherapy | Vergis et al. (2008) |

*Abbreviations are presented on the list of abbreviations, page 10.

### 4.6.1 BAX

Cell cycle-arresting tumor suppressor genes and apoptosis-regulating genes are important in the pathogenesis of prostate cancer (Visakorpi et al. 1992, Haussler et al. 1999). BAX is a soluble cytoplasmic protein that upon induction of apoptosis locates to the outer mitochondrial membrane (Wolter et al. 1997). There it forms homodimers and higher-form oligomers that resemble pores, resulting in altered permeability of the outer mitochondrial membrane (Dewson and Kluck 2009). This phase of apoptosis is referred to as the “point-of-no-return”, meaning an irreversible cascade of proteolytic events and damaged mitochondrial function. Previously, BAX overexpression was been reported in almost all prostate cancers (Krajewska et al. 1996, Johnson et al. 1998). BAX expression is also a common finding in low-grade prostatic intraepithelial neoplasia (LGPIN) and HGPINs (Johnson et al.1998). Apoptosis is a complex process, and there is a delicate balance between pro-apoptotic and anti-apoptotic proteins. For example, in a study by Mackey et al. (1998), BAX expression was not predictive as an individual factor, but an increased BCL-2/BAX ratio was associated with a poor response to radiotherapy.

### 4.6.2 BCL-2

The pro-apoptotic function of homodimerized BAX complexes may be suppressed by BCL-2, which interferes by forming heterodimers with BAX (Oltvai et al. 1993). Increased expression of BCL-2 has been reported in both LGPIN and HGPIN (Baltaci et al. 2000, Haussler et al. 1999), which are considered the most likely precursors of prostate cancer (Haggman et al. 1997, Bostwick et al. 1999, Srigley et al. 2010). The expression frequency of BCL-2 in PIN is approximately tenfold higher than expression in prostate cancers (Johnson et al. 1998). BCL-2 has been reported to be expressed more frequently in high-grade tumors by some authors (Krajewska et al. 1996, Stattin et al. 1996), whereas others have found no correlation between BCL-2 expression and tumor grade (Karaburun Paker et al. 2001). Upregulation of BCL-2 is a late step in cancer progression (Furuya et al.

1996). Overexpression of BCL-2 is associated with the transition to androgen-independent prostate cancer and poor prognosis (McDonnell et al. 1992). Increased BCL-2 expression has been noted in nodal metastases in some studies (Krajewska et al. 1996) but not in others (Tu 1996).

### 4.6.3 EZH2

Enhancer of zeste homolog 2 (EZH2) is a highly evolutionary conserved epigenetic regulator that functions as a histone methyltransferase (Simon et al. 2008). Overexpression of EZH2 has been observed in several cancers, including oral squamous cell carcinomas (Kidani et al. 2009), hepatocellular carcinoma (Sudo et al. 2005), gastric cancer (Matsukawa et al. 2006), urothelial carcinoma of the bladder (Raman et al. 2005), and prostate carcinoma (Varambally et al. 2002). EZH2 overexpression shown to have independent prognostic value in surgically treated patients (Laitinen et al. 2008). In addition, the gene encoding EZH2 is amplified in late-stage prostate cancer (Saramäki et al. 2006). One of the mechanisms by which EZH2 is believed to mediate prostate cancer aggressiveness is by transcriptional silencing of the tumor suppressor gene E-cadherin (Cao et al. 2008).

### 4.6.4 Ki-67

Ki-67 (also known as MIB-1) is one of the most extensively studied prognostic proliferation markers. It is expressed in all phases of the cell cycle in proliferating cells but not in non-dividing cells (Guillaud et al. 1989, Gerdes et al. 1991). The Ki-67 labeling index is associated with Gleason grade and independently predicts distant metastasis and cancer-related deaths (Bubendorf et al. 1998, Pollack et al. 2004). Ki-67 is an independent prognosticator in preoperative biopsies with small volumes and cancers with low Gleason score (Zellweger et al. 2009). Alternatively, a very low Ki-67 labeling index in radical prostatectomy specimens is correlated with a positive prognosis (Laitinen et al. 2008). In contrast to assessing Gleason patterns, the cell proliferation fraction as measured by Ki-67 is characterized by

minimal inter-observer variability (Gunia et al. 2008). Moreover, there are simple and rapid digital image analysis tools available to reliably measure the Ki-67 index (Tuominen et al. 2010).

### 4.6.5 MCM7

Minichromosome maintenance protein 7 (MCM7) is a critical component of the DNA replication licensing complex (Shi et al. 2008, Ren et al. 2006). In prostate cancer, proliferative indexes measured by Ki-67 are relatively low when compared to MCM7. This latter marker may therefore act as a better prognostic factor (Badmanabhan et al. 2004). In addition, the gene encoding MCM7 is amplified and overexpressed during prostate cancer progression (Shi et al. 2008, Ren et al. 2006). In our earlier study of prostatectomy-treated patients, MCM7 was a strong prognostic marker, especially in combination with EZH2 (Laitinen et al. 2008).

# AIMS OF THE STUDY

The trend toward smaller cancers presents a great diagnostic challenge for pathologists. It is well known that focal cancers restricted to a single needle biopsy usually indicate larger, bilateral and/or multifocal cancers in the subsequent radical prostatectomy specimen (Boccon-Gibod et al. 2005, Montanari et al. 2009). All diagnostic cancerous foci should be detected and reported for two reasons. First, the treatment of focal cancer in the needle biopsies may differ from that of multifocal cancers, and second, the percentage of positive cores can be used as a prognostic parameter (Winkler et al. 2004, Rajab et al. 2010).

The expression levels of apoptosis-controlling factors in benign, pre-malignant and malignant epithelium, are analyzed as possible mechanisms of multifocality in study I. Diagnostic issues and suggested resolutions associated with both small atypical foci and the risk of overlooking minute cancers are addressed in study II. Prognostic parameters, with an emphasis on the modified Gleason scoring system, are analyzed in detail in studies III and IV.

The specific aims of the present study were:

- To compare the expression of the apoptosis-regulating proteins BAX and BCL-2 in morphologically normal areas of benign and cancerous prostates
- To study the sensitivity and feasibility of routine dual-color, three-antibody immunostaining in detecting and characterizing small atypical lesions and cancers from interval sections of needle biopsies
- To evaluate prognostic histopathological parameters from needle biopsies of endocrine-treated patients
- To analyze the prognostic value of the promising molecular biomarkers EZH2, Ki-67 and MCM7 with computer-aided digital image analysis

- To compare the prognostic values of different Gleason grading methods as determined from pooled needle biopsies

# MATERIALS AND METHODS

# 1. Tissue samples

All the studies were approved by the National Authority for Medicolegal Affairs (permission 3896/32/300/02) and/or the Ethical Committee of Tampere University Hospital (TAUH) (permission R03203).

## 1.1 Study I

Cancer tissues were obtained from radical prostatectomies carried out at Tampere University Hospital in 1999 (n=33). Hyperplastic prostatic tissue was obtained from transurethral resection chips (n=9), and control tissue was obtained from autopsies of men aged 24, 26 and 47 years with no known prostatic disease (n=3). Autopsies were performed within 24 hours of death. Radical prostatectomy and autopsy specimens were completely embedded into micro- and macro-blocks. They were then cut and stained with H&E according to the normal histopathological process of Laboratory Center, Tampere University Hospital. A total of 1182 foci, including those diagnosed as benign, BPH, LGPIN, HGPIN, adenocarcinoma of various Gleason grades, and perineural invasion, were selected from H&E-stained slides and marked with a permanent pen. Selected foci were punched with a 2 mm needle and transferred to 45 tissue microarrays (TMA) (Kallioniemi et al. 2001). An individual heterogenic TMA was generated for each patient. The number of foci per individual TMA varied between 14 and 53. Benign areas were selected away from the cancer. TURP specimens were punched from the middle of the resected chips to avoid coagulation artifacts. TMAs containing 1182 different foci were immunostained with BAX and BCL-2. In addition, one H&E stain was performed for each TMA to confirm that the samples were representative.

## 1.2 Study II

Tissue was prospectively collected from 1000 consecutive patients who underwent a clinical prostate needle biopsy in the TAUH district between 2007 and 2009. Biopsies from the right and left lobes of the prostate (six tissue cores each) were formalin-fixed and paraffin-embedded into two separate blocks. Biopsies were step-sectioned at six levels in the Department of Pathology of TAUH according to normal protocols. The tissue located between the six routine sections was cut (at five levels), collected and stored at -70 °C for research purposes. For this study, stored interval sections of 200 patients were randomly chosen from among the 1000 consecutive patients. One interval section was used for H&E staining, and an adjacent section was used for 2IHC. This protocol ensured the maximal preservation of similar morphology on the stainings.

## 1.3 Studies III and IV

Approximately 1200 new patients were diagnosed with prostate cancer between 1999 and 2003 in the TAUH district. Of these, approximately 25% were primarily endocrine-treated. Patients had metastatic disease, were not suitable for radical treatment due to their general condition, or presented with low-grade cancer but wanted to have active treatment instead of active surveillance. Three cases diagnosed from TURP were excluded, and the resulting study cohort consisted of 295 consecutive, endocrine-treated patients. Original H&E-stained slides and/or paraffin-embedded specimens were available from 247 cases for study III, and 236 cases were available for study IV. Biochemical progression was the endpoint and defined as a $\geq$ 25% rise in PSA with a PSA value $\geq$ 2.0 ng/ml above the nadir in two consecutive measurements, as recommended by The Prostate Cancer Clinical Trials Working Group (PCWG2) guidelines (Scher et al. 2008). One pathologist (T. T. T.) re-evaluated biopsies for histopathological volume estimates and modified Gleason scores according to ISUP 2005 Consensus Conference guidelines. Bone

scintigraphy was performed in all symptomatic patients and in asymptomatic patients when the PSA was ≥20 ng/ml or aggressive (original compound GS >7) prostate cancer was present (Lee et al. 2000).

# 2. Immunohistochemistry

## 2.1 BAX and BCL-2 (I)

Four-micron sections of TMAs were sectioned and transferred to SuperFrost Plus glass slides. Immunohistochemical stainings were performed using a Techmate staining automat. The antibodies used were polyclonal rabbit anti-human BAX (PharMingen Europe, Hamburg, Germany) at a dilution of 1:1000 and monoclonal anti-human BCL-2 oncoprotein clone 124 (Dako, Glostrup, Denmark) at a dilution of 1:60. Antibodies were visualized with the DAB-brown reaction. The slides were lightly counterstained with hematoxylin.

## 2.2 EZH2, MCM7 and Ki-67 (II)

Immunostainings were performed with antibodies against Ki-67 (MM1, NovocastraTM Laboratories Ltd., Newcastle Upon Tyne, United Kingdom), EZH2 (NCL-L-EZH2, clone 6A1, NovocastraTM Laboratories Ltd., Newcastle Upon Tyne, United Kingdom), and MCM7 (sc-9966, Santa Cruz Biotechnology, Inc., CA) with Power Vision1TM Poly-HRP Histostaining Kit (ImmunoVision Technologies Co, Daly City, CA) according to the manufacturers' instructions. The stainings were performed with an Autostainer 480 (Lab Vision Corp, Fremont, California, USA). Briefly, the slides were autoclaved in pretreatment buffer (5 mM Tris-HCl/1 mM EDTA, pH 9) at 121°C for 2 min, followed by overnight incubation with the primary antibody diluted in pre-block solution (Ki-67 1:1,500, EZH2 1:300, MCM7 1:500). After washing and blocking, the bound primary antibody was visualized with the PowerVisionTM Poly-HRP IHC Detection Kit (ImmunoVision Technologies Corporation, Brisbane, CA). The slides were counterstained with hematoxylin.

## 2.3 Dual-color, three-antibody immunostaining with AMACR, p63 and CK34betaE12 (III)

Four-micron sections were cut and transferred to SuperFrost Plus glass slides. The slides were deparaffinized using hexane and absolute ethanol (2 x 2 minutes + 1 minute) and heat-treated in 0.01 M Tris-HCl EDTA (pH 9.0) at 121°C for 2 minutes using PickCell Antigen Retriever (PickCell Laboratories, Lelystad, Netherlands). The slides were stained using a LabVision Autostainer (LabVision, Fremont, CA). A cocktail of two mouse monoclonal antibodies against the basal cell layer, p63 Ab-4 at 1:200 (NeoMarkers, Fremont, CA) and CK-HMW Ab-3 (34betaE12) at 1:100 (NeoMarkers, Fremont, CA), and a rabbit monoclonal antibody against AMACR (clone 13H4) at 1:100 (Dako, Copenhagen, Denmark) was mixed and applied to tissue sections for 30 minutes at room temperature. After two buffer rinses, a mixture of anti-rabbit alkaline phosphatase and anti-mouse horseradish peroxidase conjugates (polymer-based secondary antibodies, Multivision kit, LabVision) were applied for 20 minutes. The peroxidase reaction was developed by first using diaminobenzidine (DAB) for 5 minutes followed by a rinse and exposure to an alkaline phosphatase substrate (HistoBlue, Multivision kit). In initial staining tests, red chromogen (Vector Red, Vector Labs, Burlingame, CA) was tested instead of HistoBlue. Following the chromogen reactions, the slides were rinsed in distilled water, counterstained with hematoxylin, and rinsed again. The slides were allowed to air dry and were coverslipped with a permanent mounting medium.

## 2.4 Microscopic evaluation of samples

### 2.4.1 Study I

The accuracy of the sampling was confirmed using one H&E-stained slide from each TMA. The TMA slides consisted of spots in rows representing benign, BPH, PIN, various grades of cancer, and foci of capsular PNI (one row for each histological type). Foci containing atrophy or inflammation or otherwise not representing the desired histology were omitted from the data analysis.

### 2.4.2 Study II

All slides were evaluated by one pathologist (T. T. T.) using an Olympus BX41 light microscope. The first evaluation was conducted using one H&E-stained interval section from each block. Histopathological diagnoses were restricted to benign, HGPIN, ASAP, or adenocarcinoma. Indications for further immunostains and a Gleason score (for adenocarcinomas) were recorded. Cases in which IHC was deemed necessary were then evaluated using 2IHC and H&E-stained sections. Simultaneous evaluation of the interval sections of tissue stained by 2IHC and H&E-stained interval sections from all blocks were performed one month later. Prior to the evaluation, the slides were randomly rearranged to hamper visual memory. In this setting, the 2IHC slides were first screened briefly using 4 x – 10 x objective lenses. The final diagnosis was assessed following evaluation of both the 2IHC and H&E-stained slides. The time required to complete the histopathological assessment was recorded for each slide. Discrepancies between these analyses and the original pathology reports were reviewed by five pathologists. A consensus was considered reached when 4 of 5 pathologists independently reached the same diagnosis.

### 2.4.3 Studies III and IV

Two slides from each patient were analyzed. The most representative H&E-stained slide, consisting of biopsies from the left or right lobe, was selected and scanned with Aperio ScanScope® XT (software version 9; Aperio Technologies, USA) and viewed in JPEG2000 using JVSview virtual microscopy software version 1.2 (Tuominen and Isola 2009). Virtual microscopy can be reliably applied to scoring prostate needle biopsies and has a strong concordance with Gleason grading (Helin et al. 2005).

The total percentage of cancer was derived from the original pathology report based on the bilateral evaluation. The greatest percentage of cancer was estimated as the proportional lengths of cancer of the total core length. The diameter of the PNI was measured from a digital image using ImageJ scale and measuring tools.

The worst (WGS) and overall (OGS) Gleason scores were evaluated according to the recommendations of the International Society of Urological Pathologists, 2005.

The OGS was derived as a sum of the predominant and most aggressive (or secondary) patterns of all biopsy cores, treated as one long core. The WGS in a single biopsy core was assessed in cases in which one biopsy contained a higher Gleason grade (e.g., 4+4 cancer) and other cores a lower grade (e.g., 3+4). In cases in which all positive biopsy cores contained the same Gleason grade (e.g., 3+3) or there was only one core positive for cancer, the WGS was equal to the OGS. A Gleason score of 7 was considered as two separate grades (e.g., the WGS could equal 4+3 and the OGS 3+4). Compound Gleason scores (CGS) were obtained from the original pathology reports from 1999-2003 such that Gleason grades were also assessed according to the recommendations prior to the refinement suggested by the ISUP 2005 Consensus Conference. The predominant the secondary Gleason patterns were estimated from two slides containing six pooled needle biopsy specimens from the right and left sides.

## 2.5 Interpretation of immunostainings (I-III)

### 2.5.1 BAX and BCL-2

Semiquantitative staining indices were determined for each focus by estimating the staining intensity (range 0-3) and multiplying it by the approximate percentage of stained epithelial cells. This method gave a staining index ranging from 0 to 300. Basal cells and luminal epithelial cells were scored as different entities. Areas with acute and/or chronic inflammation were excluded.

### 2.5.2 2IHC

Dual-color immunostainings were interpreted under an Olympus BX41 light microscope following inspection of both 2IHC- and H&E-stained slides, keeping diagnostic pitfalls in mind.

### 2.5.3 EZH2, Ki-67 and MCM7

The digitalization of the immunostained slides was performed using a Aperio slide scanner. Three hotspot areas showing the highest immunostaining were selected on a virtual microscope. Screenshots of the hotspots were captured from each slide and transferred to ImageJ, which is open access software for the analysis and processing of digital images (Collins 2007). Images were analyzed with ImmunoRatio, an analysis tool developed for analyzing nuclear immunostainings in hematoxylin-counterstained tissue sections (Tuominen VJ et al. 2010). Analysis was based on color deconvolution to separate the staining components (i.e., DAB brown and hematoxylin blue), and adaptive thresholding was used to define staining positivity. The proportion (%) of the brown-stained area over the sum of brown- and blue-stained areas was defined as the labeling index. Results of the automated analysis were verified by one pathologist (T. T. T.), who compared the original image to the segmented image.

# 3. Statistical analyses (I-IV)

## 3.1 Study I

Basic characteristics of the staining indices were examined by cross-tabulation. A linear mixed-models analysis, in which staining indices were considered continuous, was used in paired comparisons between all morphological categories. The BAX and BCL-2 staining indices were considered dependent variables; morphological classification was the explanatory variable. The data were fitted to a random-effects model with individual patients as the random effect. To further analyze immunoreactivity in controls, tissue from patients with BPH and normal epithelia from prostate cancer patients (CaNE) were examined. For these analyses, we used a chi-squared test, in which the staining indices were dichotomized using a value of 50 as a cut-off point between negative and positive. A P-value 0.05 was considered the limit of statistical significance. Statistical analyses were performed using SPSS versions 11.0 and 11.5 and SAS version 8.1.

## 3.2 Study II

The Wilcoxon signed rank test was used for paired comparisons between the intraobserver settings to evaluate statistical differences in the time required to complete the histopathological assessments. Statistical analyses were performed with GraphPad Prism version 4.00. In addition, reproducibility of Gleason scoring was manually analyzed using the kappa method.

## 3.3 Study III

Fisher's exact, chi-squared and one-way ANOVA tests were used to evaluate associations between the variables. Survival analysis was performed using the Kaplan–Meier method, and the statistical significance of survival differences between patient groups was determined using the Mantel–Cox test. Univariate and multivariate Cox regression analyses were performed to calculate the relative risk estimates (RR) and to evaluate the independence of the prognostic markers. Statistical analyses were performed with BMDP.

## 3.4 Study IV

The agreement between Gleason scoring methods was analyzed with the κ-coefficient method. Survival analyses were performed using the Kaplan–Meier method, and the statistical significance of survival differences between patient groups was determined with a Mantel-Cox (log-rank) test. Univariate and multivariate Cox regression analyses were performed to calculate the relative risk estimates (RR) and to evaluate the independence of the prognostic grading methods. Statistical analyses were performed with BMDP.

# RESULTS

# 1. Expression of apoptosis regulators in heterogenic TMAs (I)

## 1.1 BAX

In autopsy samples from normal (control) prostates, no BAX expression was observed on the luminal epithelium. However, BAX was expressed in basal cells, thus serving as an intrinsic staining control. Normal luminal epithelium showed elevated BAX expression in 16.3% of cancerous prostates and in 3.9% of BPH cases ($p<0.001$). In paired comparisons, BAX expression was significantly lower in adenocarcinomas of Gleason grade 2 than in Gleason grades 3-5 ($p<0.001$). Both the staining index estimate of BAX expression (mean 127, 95% confidence interval 120-134) and the number of BAX-positive foci (91%) were significantly higher in PNIs than that in any other group ($p<0.001$ to $p<0.009$). In conclusion, BAX expression showed nearly steadily increasing staining indices from benign luminal epithelium to more aggressive patterns of cancer and PNI.

## 1.2 BCL-2

In contrast to the lack of BCL-2 expression in control prostates, the normal epithelium of cancerous prostates showed elevated BCL-2 expression in 6.9% of foci ($p=0.004$). BCL-2 expression was most frequent in foci with LGPIN (35%) and HGPIN (24%). In well-differentiated cancers of Gleason grades 2 and 3, BCL-2 expression was virtually absent. However, BCL-2 expression was detected in approximately 13% of high-grade cancers of Gleason grades 4 and 5 and in approximately 12% of foci with perineurally growing cancer.

# 2. Dual-color immunostaining in detecting small cancers (II)

Our initial staining tests included dual-color stainings with brown-blue and brown-red combinations and conventional single-chromogen DAB-brown immunostaining reactions for AMACR. Both dual-color stainings showed satisfactory results. However, because brown-blue was considered easy to interpret and because color deconvolution of brown-red digital images could be more difficult, the former was chosen for this study. Examples of the stainings are presented in Figure 12.

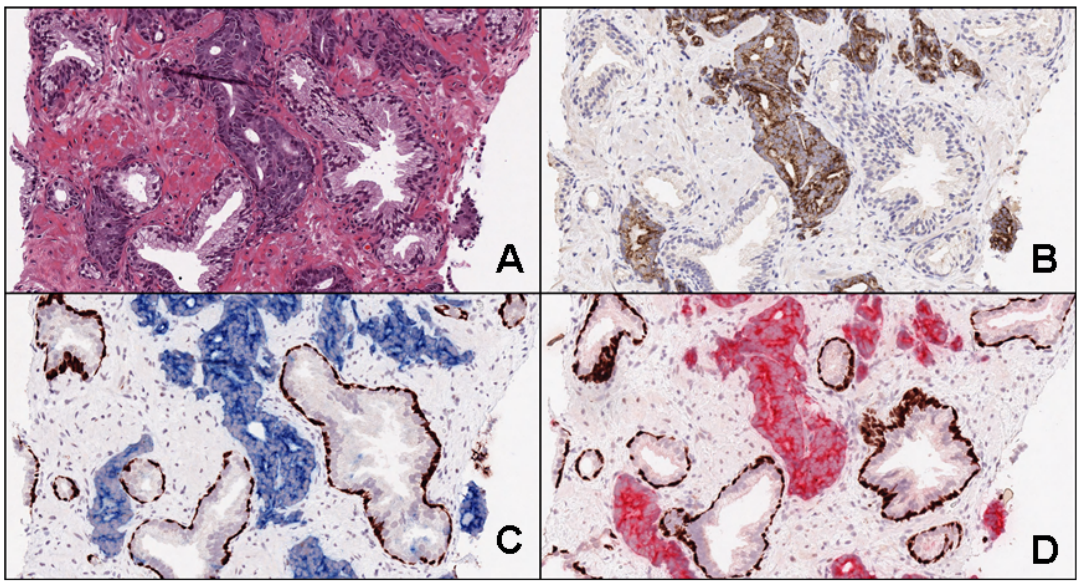


Figure 12. An example of a small focus containing poorly differentiated adenocarcinoma of GS 4+4=8. The sample shows fusion of glands and infiltrative growth between obviously benign glands. A) Cancer is readily detectable in the H&E staining. B) Single-chromogen immunostaining against AMACR (DAB-brown). C) Three-chromogen immunostainings against basal cells (p63 and CK34betaE12) and AMACR. Basal cells visualized with DAB-brown and AMACR with HistoBlue. D) Same as C except that HistoBlue is substituted for Vector Red. (100 x).

## 2.1 Increased sensitivity

Of the 200 randomly selected patients, adenocarcinoma was diagnosed in 87 (43%) patients in the original pathology reports. H&E and occasionally immunostainings (on request) were used for these diagnoses. Of the 113 cases diagnosed as non-malignant, 14 additional putative cancers were identified from interval sections with the aid of routine 2IHC by one pathologist (T. T. T.). Interval sections from 14 cases, including one H&E staining and one 2IHC, were then independently reviewed by five pathologists. Retrospective re-evaluation yielded 8 additional consensus diagnoses of adenocarcinoma and one ASAP. In 5 cases, a consensus was not reached. Six of the eight consensus cancers were well-differentiated (GS 3+3=6), while two (25%) were graded GS 4+4=8.

## 2.2 Feasibility analysis

The amount of time spent on the microscopic assessment of H&E and 2IHC (average 251 sec.) staining was shorter than for H&E followed by 2IHC on request (average 299 sec., $p<0.0001$). The microscopic assessment of H&E and 2IHC was faster than for H&E because the risk of overlooking small atypical lesions on 2IHC was considered smaller. Although the evaluation of the biopsies was faster, more lesions were found, and a good reproducibility of Gleason scores was achieved, with a kappa-value of 0.72.

# 3. Prognosticators in the needle biopsies of hormone-treated patients (III and IV)

## 3.1 Basic characteristics of the studied patients

The median age of the patients at the time of diagnosis was 73.6 years (range 52.7-88.8 years). The median follow-up time was 56.5 months (range 8-104 months). The median pre-treatment PSA value was 16.5 ng/ml (range 1.9-10750 ng/ml). Over half (54.9%, 162/295) of patients had localized disease (cT1-T2). One third of patients (33.6%) experienced PSA progression. Cancer-specific mortality was 6.4% (19/295), and deaths due to other reasons occurred in 25.7% of patients (76/295). The distribution of primary hormonal treatments was the following: LHRH analog (n=208); surgical castration (n=55); antiandrogen bicalutamide (n=27); and maximal androgen blockade (n=3).

## 3.2 Histopathological parameters and proliferation markers (III)

All tumor histopathologic parameters were significantly associated with progression-free survival based on univariate analyses (TABLE III). Of these, the percentage of cancer-positive cores showed the strongest prognostic value, with a relative risk of 3.2 (95% confidence interval 1.9-5.2). The only histopathological parameter not associated with progression was the diameter of the greatest nerve showing PNI ($p$=0.75). All immunohistochemical proliferation markers were also significant prognosticators based on univariate analyses (TABLE III).

**TABLE III – univariate analysis of prognostic markers**

| Parameter[1] | RR | 95% CI | *p-value* |
|---|---|---|---|
| PSA (ng/ml) | 5.6 | (3.6 - 8.7) | <0.0001 |
| cT-stage | 4.6 | (3.0 - 7.2) | <0.0001 |
| WHO grade | 3.3 | (2.3 - 4.8) | <0.0001 |
| Metastasis | 3.2 | (2.1 - 4.9) | <0.0001 |
| Ki-67 IHC | 3.4 | (2.1 -5.5) | <0.0001 |
| Reclassified Gleason score | 2.6 | (2.0 - 3.6) | <0.0001 |
| Cores positive for cancer | 3.2 | (1.9 - 5.2) | <0.0001 |
| Gleason score | 2.6 | (1.8 - 3.6) | <0.0001 |
| Perineural invasion | 2.2 | (1.7 - 3.0) | <0.0001 |
| The total percentage of cancer | 2.1 | (1.6 - 2.8) | <0.0001 |
| The greatest percentage of cancer | 2.1 | (1.5 - 2.8) | <0.0001 |
| MCM7 IHC | 2.4 | (1.5 - 3.9) | <0.0001 |
| EZH2 IHC | 2.0 | (1.2 - 3.3) | 0.004 |

[1]cut-off values, PSA ≤20, >20 ng/ml, cT-stage:cT3-4 against cT1-2, WHO grade: I, II, III, Metastasis: M+ against M0, Ki-67:≤5%, >5-10%, >10%, reclassified Gleason score: 5-7(3+4), 7(4+3)-8, 9-10, cores positive for cancer: <75%, 75-100%, Gleason score: <7, 7, >7, perineural invasion: 0, 1-3, >3, the total percetage of cancer: ≤10%, >10-40%, >40%, the greatest percentage of cancer in a single biopsy: ≤30, >30-80%, >80%, MCM7: ≤20%, >20%, EZH2: ≤15%, >15%.

In a first multivariate analysis of the 12 parameters shown in TABLE III (reclassified GS excluded), four independent prognostic markers were identified:

- Pre-treatment PSA level (2.6, 1.3-4.9)
- Gleason score (2.1, 1.4-3.2)
- Perineural invasion (1.6, 1.2-2.2)
- Clinical T stage (2.0, 1.1-3.9)

Because samples with GS 3+4=7 and GS 4+3=7 had clearly different prognoses, a second multivariate analysis was performed with the non-conventional, "reclassified" Gleason score groups 5-7(3+4), 7(4+3)-8, and 9-10. The same four prognosticators were identified in the second multivariate analysis, with the Gleason score now being the strongest prognostic factor:

- Gleason score (2.2, 1.5-3.2)
- PSA level (2.1, 1.1-4.2)
- PNI (1.6, 1.2-2.2)
- Clinical T stage (1.9, 1.0-3.7)

To further evaluate the prognostic abilities of the markers, various models that included the four independent factors were created and tested with different cut-off values for Gleason scores. Combinations of the independent markers (PSA>20 or GS≥4+3 or PNI>3 or cT>2) yielded the best risk stratification (RR 11.6, 10.4-12.7). Patients with >3 foci with PNI were at high risk for early progression. Within the subgroup of patients with Gleason score 7, the progression-free time for patients with Gleason grading 4+3 was significantly shorter than for patients with grading 3+4 ($p=0.013$). In the same patient group, Ki-67 expression with a cut-off value of 10% was highly capable of identifying patients with early PSA recurrence ($p<0.0001$).

## 3.3 Comparison of prognostic abilities of worst and overall (modified) Gleason scores (IV)

The average number of core biopsies from one lobe was 4.5 (median 4, range 1-9), and the average number of positive biopsy sites was 3.1 (median 3, range 1-7). The number of cases with multiple positive biopsy sites was 191.

A clear trend towards higher Gleason sums was noted from compound Gleason score (CGS) to OGS and from OGS to WGS. In the original pathology reports from 1999-2003, CGS of ≤5 were assessed in 22 patients. With the same needle biopsy material but using the modified Gleason score system, WGS=5 and OGS=5 was assessed in only four patients, and no patients had a GS below 5.

The WGS was higher than the OGS in 43 (18%) cases. In 14 of 65 cases with OGS=7, at least one biopsy core containing higher-grade cancer (WGS 4+4=8) was found. In 12 of 39 cases with OGS 3+4=7, the biopsy core containing the highest Gleason score yielded WGS 4+3. The difference between WGS and OGS was 2 in three cases. In all of these cases, the OGS was 8 (3+5 or 5+3), and the WGS was 10 (5+5). The agreement between the WGS and OGS was high (κ-coefficient=0.82). When compared with the original CGS, which was obtained using pathology reports generated before the refined guidelines, a significantly lower concordance was

found between the WGS and the CGS (κ=0.48) and between the OGS and the CGS (κ=0.44).

Univariate analyses of OGS and WGS yielded similar RRs. Re-classification of the Gleason score groups to <7(4+3), 7(4+3)-8, and 9-10 slightly improved the prognostic value of both the WGS and OGS. In multivariate analyses of the six different Gleason grading methods, reclassification of the OGS as <7(4+3), 7(4+3)-8, and 9-10 was the strongest (and only) independent prognostic factor (RR 2.6, 95%-confidence interval 2.0-3.5).

# DISCUSSION

Pathology has a long tradition on analyzing thin-cut histochemically-stained tissues under a light microscope. During the last two decades, the significance of immunohistochemistry has increased and broadened the search for new diagnostic and prognostic tools for use in pathology. In the histopathology of the prostate gland, there are several diagnostic pitfalls to keep in mind. For instance, pre-malignant lesions and adenocarcinoma must be distinguished from benign glands, AAH, atrophy, and seminal vesicle epithelium. Immunohistochemistry has proven to be an invaluable tool in aiding differential diagnostics. However, a qualified laboratory process with strong reproducibility is required for reliable interpretation of the immunostainings.

Digitalized images of immunostainings have enabled the development of automated image analysis tools, which can yield better objectivity and reproducibility. Microscopic assessments of tissue histology are important parts of all immunohistochemical studies because tissues are visually chosen for analysis. In addition, correct interpretation of the results of automated analysis also requires comparisons of the morphologies of different stainings. All of the studies presented here combined detailed, “old-fashioned” histopathological assessment with more modern tools, including automated image analyses, immunohistochemical stainings and virtual microscopy.

# 1. Androgens, apoptosis and field effect (I)

The prostate and seminal vesicles are accessory reproductive glands, located adjacent to one another, but are still quite different in terms of malignant potential. The former suffers from multifocal cancers with extremely high incidence, while there have been only approximately 50 case reports of primary carcinomas reported world-wide for the latter (Thiel and Effert 2002). The reason behind this phenomenon is incompletely understood. Development of the normal prostate and prostate cancer is regulated by androgens (Isaacs et al. 1992), and androgen deprivation (including finasteride) induces apoptosis of prostatic epithelial cells (Kyprianou et al. 1990, Golbano et al. 2008). However, castration induces apoptosis in the seminal vesicle epithelium in a similar manner (Tanji et al. 2003).

We studied apoptosis as one possible mechanism underlying the multifocality of prostate cancer. The key apoptotic regulators BAX and BCL-2 showed increased expression in some foci of normal epithelium of cancerous prostates, suggesting altered apoptotic control. These immunohistochemical changes may represent the very early phase of the multifocal carcinogenetic process. The concept of the field effect, also termed field cancerization, was introduced in the 1950s. At this time, authors noted that microscopic abnormalities in grossly benign tissues were associated with an abnormally high risk of a recurrence of multifocal oral squamous cancer (Slaughter et al. 1953). The concept of the field effect has extended through the development of molecular biomarkers, including GSTP1, APC and RARb2 (Chai and Brown 2009). To date, abnormal expression of such field effect biomarkers in normal tissues have been reported in various organs, including the prostate (Nonn et al. 2009). In addition, invasive cancer communicates with its environment, the stroma, leaving detectable molecular fingerprints (Halin et al. 2010). In our study, benign foci of cancerous prostates in TMAs were consciously sampled away from the cancer. Thus, the observed changes in apoptotic regulation

are more likely to represent the field effect than tumor-induced secondary changes and may explain the multifocal origin of prostate cancer.

Most newly detected prostate cancers in the future will have a small volume and therefore will not always be observed with the current diagnostics based on needle biopsies. The tissue adjacent to cancer has been shown to harbor morphological or genetic changes (Malins et al. 2004, Risk et al. 2010). Additionally, cancer can induce detectable changes to its environment to grow and invade (Halin et al. 2010). For these reasons, benign tissue surrounding the tumor has recently earned interest as a potential diagnostic and/or prognostic source of biomarkers (Halin et al. 2010). The concept of tumor-indicating normal tissue, resulting from secondary responses of the microenvironment, is a rather new subject (Halin et al. 2010) and needs to be distinguished from the carcinogenic field effect (Nonn et al. 2009), which is more likely to represent a primary change underlying prostate cancer multifocality. Secondary responses adjacent to the cancer could be most useful in the context of diagnostics, while biomarkers associated with the field effect could be useful in terms of cancer prevention, surgical considerations and prognosis (Chao and Brown 2009). Clinical applications are awaited.

The speed of tumor growth is dependent on the proliferation rate and average lifetime of the cancer cells (Isaacs et al. 1992). Cell proliferation rates increase in more aggressive prostate cancer phenotypes (Häussler et al. 1999). According to our results, apoptotic control is likely to be involved in and modulated during local progression of prostate cancer. Furthermore, our results demonstrate that the ratio of pro- to anti-apoptotic proteins is variable in different steps of progression. BCL-2 expression has been noted in 35% of HGPIN cases, in 2-25% of prostate cancers (with a higher tendency in high-grade cancers), and in 38% of nodal metastases of prostate cancer (Krajewska et al. 1996, Johnson et al. 1998, Häussler et al. 1999). Moreover, concomitant upregulation of other anti-apoptotic proteins, such as members of the inhibitor of apoptosis (IAP) protein family, occurs early in the development of prostate cancer (Krajewska et al. 2003). In our study, BCL-2 expression was noted in the premalignant lesions of LGPIN (35%) and HGPIN (24%) samples. Low-grade tumors were completely negative for BCL-2, but expression was apparent in approximately 12-13% of high-grade cancers (GS≥8) and in PNIs. Moreover, BAX expression was highest in foci with PNI. Prostate cancers not only express neurotrophins and their receptors but also can induce

neural growth, neurogenesis and axonogenesis (Ayala et al. 2008). Increased proliferation rates together with reduced apoptotic indices have been noted in PNI (Yang et al. 1996). Apparently, the effect of pro-apoptotic BAX is overridden by multiple mechanisms in PNI foci.

Prostate cancer can eventually escape androgen-regulated apoptotic control. Various mechanisms in the transition to hormone-refractory disease have been demonstrated, including the amplification of androgen receptor (AR), AR mutations, and the induction of neuronal apoptosis inhibitory protein (NAIP) by androgen deprivation (Visakorpi et al. 1995, Linja et al. 2001, Haapala et al. 2007, Chiu et al. 2010). To date, there are no efficient treatment options for CRPC.

# 2. Diagnostic immunohistochemical markers (II)

PSA screening can detect smaller and better-differentiated cancers (Laurila et al. 2009, Cremers et al. 2010). The consequences of our results demonstrating the increased sensitivity of 2IHC in detecting small cancers from needle biopsies are difficult to estimate. Even the definition of minute cancer is difficult, resulting in interobserver variation among uropathologists (Van Der Kwast et al. 2010). In a study by Wolters et al. (2010), a diagnostic delay of four years did not the affect patient prognosis. Furthermore, preliminary results of active surveillance show that well-differentiated small-foci prostate cancers have a favorable prognosis and that intervention is rarely needed (Klotz et al. 2006). Although most of the additionally detected small cancers may be clinically insignificant, patients need to be monitored frequently, keeping in mind that most cancers are bilateral and multifocal (Boccon-Gibod et al. 2005, Montanari et al. 2009). In our study, comparing routine 2IHC and clinical reports, two of eight of cancers detected with the aid of 2IHC were high-grade (GS 4+4=8). In addition, two cases (1%) of high-grade adenocarcinoma were overlooked by one pathologist (T. T. T.) on the first examination with one H&E staining and one optional 2IHC slide compared to the routine 2IHC + H&E protocol. In contrast to our results, Pavlakis et al. (2010) found only one overlooked cancer and one ASAP among 250 patients whose needle biopsies were routinely immunostained with p63, CK34betaE12, and AMACR. Moreover, Paner et al. (2008) concluded in a review article that no routine immunostainings are required. Due to the short follow-up time, no prognostic comparisons between routine 2IHC and current practices can be made.

Clearly, patients with aggressive disease but with minimal findings in needle biopsies need to be identified to receive adequate treatment. In some cases, routine 2IHC may yield detection of clinically insignificant cancers, and there is a risk of overtreatment. For the pathologists, however, improved sensitivity indicates better

diagnostic quality. In addition, we ensure the equality of patients by performing 2IHC non-selectively on samples from all patients.

In laboratory medicine, improved sensitivity is frequently achieved at the expense of decreased specificity. In the present study, 14 putative cancers were found in new patients. Of these, the consensus of five pathologists (defined as agreement of 4/5) yielded 8 cancer diagnoses, one ASAP, and five cases in which no consensus was found. The eight cancers diagnosed by consensus will be treated as carcinoma according to their prognostic parameters. The only consensus case of ASAP will likely be submitted to repeated biopsies in addition to frequent PSA monitoring. The five cases in which no consensus was reached may represent false positives. The study by Pavlakis et al. (2010) found a substantial number of benign glands negative for the basal cell markers p63 and CK34betaE12, potentially representing false positives. In our study, however, three of the five cases without consensus were submitted to re-biopsies, and all were identified as GS 3+3=6 cancer. Because of this result, we believe that routine 2IHC is valuable in pinpointing small neoplastic lesions.

Given our results that routine 2IHC increases the detection of small cancer foci, multifocal cancers should also be more easily detected, affecting prognostic parameters from needle biopsies e.g. number of positive cores. Some patients with focal cancer observed on H&E staining would be given a cancer diagnosis based on multiple cores if 2IHC was performed and would receive active treatment instead of surveillance. According to the current national guidelines in Finland (Suomalainen Lääkäriseura Duodecim ja Suomen Urologiyhdistys ry. at http://www.kaypahoito.fi/web/kh/suositukset/naytaartikkeli/tunnus/hoi11060#s8 25.02.2011), the number of actively treated patients would likely increase if more cancer-positive cores were found. These consequences need to be monitored in a longer follow-up study.

The number of cases with ASAP as the worst diagnosis was also increased following our protocol, the majority of which would have been diagnosed as benign without immunohistochemistry. It is difficult to estimate the significance of this finding, but due to clinicians' awareness of these cases, some cancers may be detected earlier.

In general, dual-color immunostainings were considered technically successful and easy to interpret. In approximately 15% of obvious cancer cases that were

negative for basal cell markers, immunostaining against AMACR was weak and/or patchy. This result may reflect the biology of prostate cancer, but technical failure cannot be excluded. In previous reports, the frequency of compromised staining of AMACR has been similar, between 0% and 18% (Jiang et al. 2001, Zhou et al. 2004, Browne et al. 2004, Jiang et al. 2005).

Routine 2IHC staining of all prostate biopsies was associated with diminished microscopy time. One explanation for this efficiency can be deduced from the study protocol, in which the 2IHC was first briefly screened. After screening the 2IHC slide, the risk of missing a minute cancer is lower. The interpretation of 2IHC-stained slides could be performed so rapidly because the blue stain is readily distinguished from the background when scanning the slides with a 4 x objective. As expected, this stain served as an alarm. Additionally, 2IHC was helpful in differential diagnostics, making decision-making more efficient.

The costs of the 2IHC protocol must be weighed against the benefit of finding more cancers. If we compare only the reagent costs of routine 2IHC (50 € per cancer or 600 € per detected additional cancer), for instance, to the costs of oncological or surgical treatments, the costs do not seem to be extremely high provided that the cancer incidence in the needle biopsies remains high. The total exact costs of routine 2IHC are difficult to estimate because this protocol would likely:

- increase the number of cancer diagnoses
- increase the number of ASAP diagnoses, leading to repeated biopsies
- resolve most ASAP diagnoses from H&E to benign or malignant
- resolve approximately half of the HGPIN diagnoses based on H&E staining to either benign or malignant
- influence prognostic parameters and thereby affect the chosen treatment
- decrease the microscopy workload of the pathologist
- increase dictation time

We consider that the costs of routine 2IHC may be tolerable, at least in the case of six biopsies from one “lobe” of the prostate embedded in the same paraffin block. However, individually embedded needle biopsies would yield six-fold higher costs, which may be too high for routine diagnostics. In conclusion, considering that the quality of treatment is as good as the quality of diagnostics, the routine use of 2IHC could be tested in clinical use, at least for pooled (6+6) biopsies.

# 3. Prognostic factors in needle biopsies (III, IV)

Pre- and post-treatment nomograms are used to predict patient outcome. Indeed, many nomograms have been created for all recommended treatment forms (D'Amico 1999, Kattan et al. 1999, Kattan et al. 2001, Smaletz et al. 2002, Makarov et al. 2007, Zelefsky et al. 2007, Spiess et al. 2010). There are also numerous prognostic studies of histopathological parameters of needle biopsies from surgically treated patients (Nelson et al. 2002, Winkler et al. 2004, San Francisco et al. 2004, Villamon-Fort et al. 2007, Brimo et al. 2008, Rajab et al. 2010). Integration of the sum of the complex data available from individual studies has yielded simple online nomograms meant to aid clinicians in decision-making. These include, for example, nomograms for different treatments of prostate cancer at the Memorial Sloan-Kettering Cancer Center (MSKCC at http://www.mskcc.org/applications/nomograms/prostate/index.aspx 1.3.2011) and a recently published risk calculator by Katz et al. (2010) (http://www.capcalculator.org 1.3.2011).

Several parameters reported from core biopsies are considered to be predictive rather than prognostic factors and correlate to findings from the radical prostatectomy specimen. Prognostic factors may differ in other treatment forms, including ADT, in which clinicians need to rely on the biopsy material only. A nomogram for ADT-treated, non-CRPC patients was recently created, but complementary histopathological parameters were not included (Cooperberg et al. 2009). Although ADT is used most frequently in advanced disease, it has been used for elderly men who are not suitable for intent-to-cure therapies but desire to be actively treated instead of following a course of watchful waiting. Because no radical therapy had been applied in our patient cohort, this patient sample allowed for a unique prognostic study.

The strongest prognostic factors in the endocrine-treated cohort studied with prostate needle biopsies were GS, pre-treatment PSA value, multiple PNI, and cT

stage. Anatomically, nerve bundles are located near the capsule of the prostate, and perineural growth indicates the minimum subcapsular infiltration of cancer. Moreover, the most common mode of local spreading of prostate cancer through the capsule occurs inside the perineural space (Villers et al. 1989). Because PNI was identified as an independent prognosticator and not only a surrogate marker for cT stage, perineurally growing cancer may receive a growth advantage from neural paracrine factors. This growth advantage may then interfere with apoptotic control (Yang 1996). Recently, Chiu et al. (2010) described a survival mechanism for prostate cancer cells in endocrine-treated patients. Androgen deprivation therapy induces the expression of NAIP, which belongs to a larger family of IAP proteins. Neural expression of NAIP may lead to apoptotic escape from of cancer cells in vivo and possibly explains the increased risk for biochemical recurrence in hormone-treated patients with PNI. Unfortunately, no literature regarding NAIP expression in peripheral nerves could be found.

Two trends regarding the detection and diagnosis of prostate cancer have occurred over the last two decades: i) cancers are detected earlier, when they are smaller and better differentiated, but ii) they are graded as more aggressive than in the past due to grade inflation (Albertsen et al. 2005a). Despite recent modifications of GS scoring and the Will Rogers phenomenon (Thompson et al. 2005), the GS remains the most powerful prognosticator in prostate cancer (Epstein et al. 2005, Helpap and Egevad 2006). As a result of the modification of the Gleason grading system, the cut-off between low-grade and high-grade cancer may have shifted upwards (Helpap and Egevad 2009, Stark et al. 2009). A similar phenomenon was observed also in our study, in which non-conventional grouping of Gleason scores (GS 3+4 vs. 4+3) gave the best prognostic values. Similar improvements were additionally noted in both the WGS and OGS methods of assessing Gleason scores. In a multivariate analysis, OGS was the only independent prognostic factor of the six different grading systems (both conventional and reclassified versions of WGS, OGS and CGS). However, in univariate analysis, WGS performed slightly better than OGS, with a relative risk of 2.8 (2.5-3.1) compared to 2.6 (2.4-2.9) for OGS. Relative risks from a three-category variable may be difficult to determine, but we conclude that both scoring systems offer similar information regarding patient prognosis. Lotan and Epstein (2010) examined needle biopsy studies and reported that the prognostic cut-off between low-grade and high-grade cancer shifted from

Gleason 6 vs. 7 to Gleason 3+4 vs. 4+3 due to the revised ISUP guidelines. This conclusion was also suggested by prostatectomy specimens studied by Helpap and Egevad (2008). We confirm this phenomenon for needle biopsy material, at least in our unique, endocrine-treated cohort. If we consider the impact of the modified GS, some cases will have a GS 3+4=7 instead of a GS 3+3=6. Logically speaking, cases with a GS 4+3=7 remain unchanged, making this category more resistant to grade inflation, emphasizing its prognostic value.

Cell proliferation markers have the best prognostic value among biomarkers for prostate cancer but are still not superior to the Gleason sum. Due to the established problems of Gleason grading, including low reproducibility and high interobserver variability, biomarkers could potentially have additive value in Gleason score subgroups. Several earlier studies have shown that the fraction of proliferative cells is useful in risk assessment (Visakorpi et al.1991, Bubendorf et al. 1998, Pollack et al. 2004, Laitinen et al. 2008, Zellweger et al. 2009). In the present study, a Ki-67 immunostaining index over 10% was capable of detecting patients with a high risk for progression. The modified GS was very strong prognosticator on the both ends of the scale, but GS 7 was assessed in 32% patients, indicating moderate risk. These patients could be further substratified by GS 3+4 vs. 4+3 to low- vs. high-risk groups, respectively. An important and clinically relevant observation was that Ki-67 was especially useful in identifying patients with aggressive disease within the moderate risk group with GS 7. In conclusion, IHC for Ki-67 is routinely available in virtually all pathology laboratories, and its use, for example with a cut-off value of 10 %, could be encouraged for intermediate risk patients (GS 7).

Our previous results with MCM7 and EZH2 suggested that these biomarkers were independent prognostic factors in surgically treated patients, especially when their expression indexes were combined (Laitinen et al. 2008). In the present study of endocrine-treated patients, both biomarkers were significant predictors in the univariate analysis. However, we were unable to repeat our earlier findings in the multivariate analysis. All studied variables, including morphological biopsy parameters, were associated with each other. Moreover, the prognostic abilities of these variables in the univariate analysis were rather similar (TABLE III). When many good prognosticators are identified, small differences and coincidence may play a role in their perceived importance. In addition, there are several other differences that could explain discordant findings between the two studies, including

materials (core biopsy vs. radical prostatectomy) and methods (manual vs. automated counting). Furthermore, the modified recommendations with respect to Gleason grading may have led to a better prognostic value of the GS.

# 4. Towards better diagnostics in the PSA era (II, IV)

The processing of prostate biopsies is variable and debated. A recent multicenter questionnaire by ENUP showed that slightly more than half of European centers utilize individual embedding of needle biopsies, while other centers embed 2-6 biopsies in the same paraffin block (Lars Egevad 2011, personal communication). The embedding procedure influences Gleason scores with respect to whether the worst or overall GS is given and, therefore, the choice of treatment. Individual worst Gleason scores were recommended by the ISUP 2005 Consensus Conference if the biopsies are immersed in separate formalin containers and embedded individually (Epstein et al. 2005). The use of individual embedding may yield less fragmented needle biopsy tissue cores but is more laborious.

Another disadvantage of pooled biopsies is the loss of site information. Information regarding the location of the focus would be essential when considering local therapy (e.g., targeted brachytherapy or cryotherapy) in focal carcinomas (Eggener et al.2007, Karavitakis et al. 2010). Moreover, the anatomic localization of carcinoma foci is useful when planning nerve-sparing radical prostatectomy and for avoiding side effects of external-beam radiotherapy. Although there is a significant degree of variation between laboratories, only four studies comparing predictive or prognostic abilities of worst and overall Gleason score have been published (Forman et al. 2000, Kunz and Epstein 2003, Poulos et al. 2005, Kunju et al. 2009). Of these, only the study by Kunju et al. (2009) was performed using the modified Gleason system.

Embedding of multiple biopsies in the same paraffin-block is not recommended by the ISUP because of assumed fragmentation and technical difficulties in flattening the biopsies (Epstein et al. 2004, Epstein et al. 2005). However, these presumptions have not been thoroughly studied. The median length of the biopsies in the European Randomized Study of Screening for Prostate Cancer was 56.3 mm, and the length of glandular tissue was 44.6 mm (Wolters et al. 2008). Based on our

experience of consultation biopsies from other institutes, there are no major differences in the length or quality of individual, double-embedded or pooled needle biopsies.

*Future directions*

Optimal methods for embedding pooled biopsies are needed to facilitate the important application of routine 2IHC, the costs of which would otherwise be prohibitive. We are currently testing two recent innovations, engraved paraffin blocks (Tolonen et al., manuscript under preparation) and the Paraform® Sectionable Cassette System from Sakura (Dimenstein IB 2010) to:

- maintain locus information of the biopsies,
- flatten them properly
- enable 2IHC stainings from one (6 biopsies) or both sides (12 biopsies) simultaneously

The quality of individually embedded biopsies vs. pooled biopsies will be compared by measuring their areas with the aid of digital image analysis. The development of universally acceptable technical solutions for pooled biopsy protocols will gradually facilitate the routine use of 2IHC. In turn, this advance will hopefully improve the diagnostic quality of random needle biopsies of the prostate gland in the post-PSA era.

Due to intra- and interobserver variability problems associated with Gleason grading, our group is also developing an automated analysis system of 2IHC-stained prostate biopsies. The color components of the digitalized image of the 2IHC staining (blue and brown) are relatively easy to separate by color deconvolution and thresholding (Ruifrok and Johnston 2001). Previously, texture analysis of prostate biopsies has been challenging, but it may be possible through digitalized 2IHC images following initial editing of images by deconvolution and thresholding. Current applications of texture analysis, such as local binary pattern (LBP) (Ojala et al. 1994), are able to recognize patterns that were previously considered difficult. For example, face-recognition using LBP is already incorporated in common photo library software and cameras. Texture analysis of digitalized 2IHC images will hopefully yield a more reproducible method for assessing Gleason patterns in the near future.

Until computer-based analysis of prostate biopsies is a reality, pathologists will need to perform the scoring. Consistent scoring between pathologists is achieved with the aid of reference figures. Both the old and new “official” figures of the Gleason patterns (Figure 5, pp. 31 and Figure 6, pp. 33) need to be updated. Both suffer from major errors due to modified recommendations (Epstein 2010). To resolve this problem, our preliminary testing with digital images of 2IHC slides resulted in a new diagram (Figure 13). All cancer patterns were cut from digitalized images of 2IHC slides and thus represent real (i.e., non-stylized) adenocarcinomas. To generate the black and white schema, the cancer pattern images were converted to binary mode, thresholded and median-filtered in ImageJ. Some details have been manually painted. We have also attempted to eliminate the overlapping of patterns observed on several grades in earlier versions by Gleason (Figure 5, pp. 31) and Brunbaugh (Figure 6, pp. 33). The key changes are:

- Grade 1 does not exist.
- Grade 2 consists of tightly packed round glands with some variation in size and shape. No infiltrative patterns are accepted.
- All infiltrative cancer, that forms separate glands are included in grade 3.
- Small glands with lumens are also included in grade 3.
- Small glands without lumens are included grade 4.
- All cribriform patterns are included in grade 4 (except if with comedonecrosis, which is grade 5)

With some minor refinements, Figure 13 is our proposal for a new reference figure of Gleason patterns and should be consistent with the current recommendations of grading prostatic adenocarcinoma.

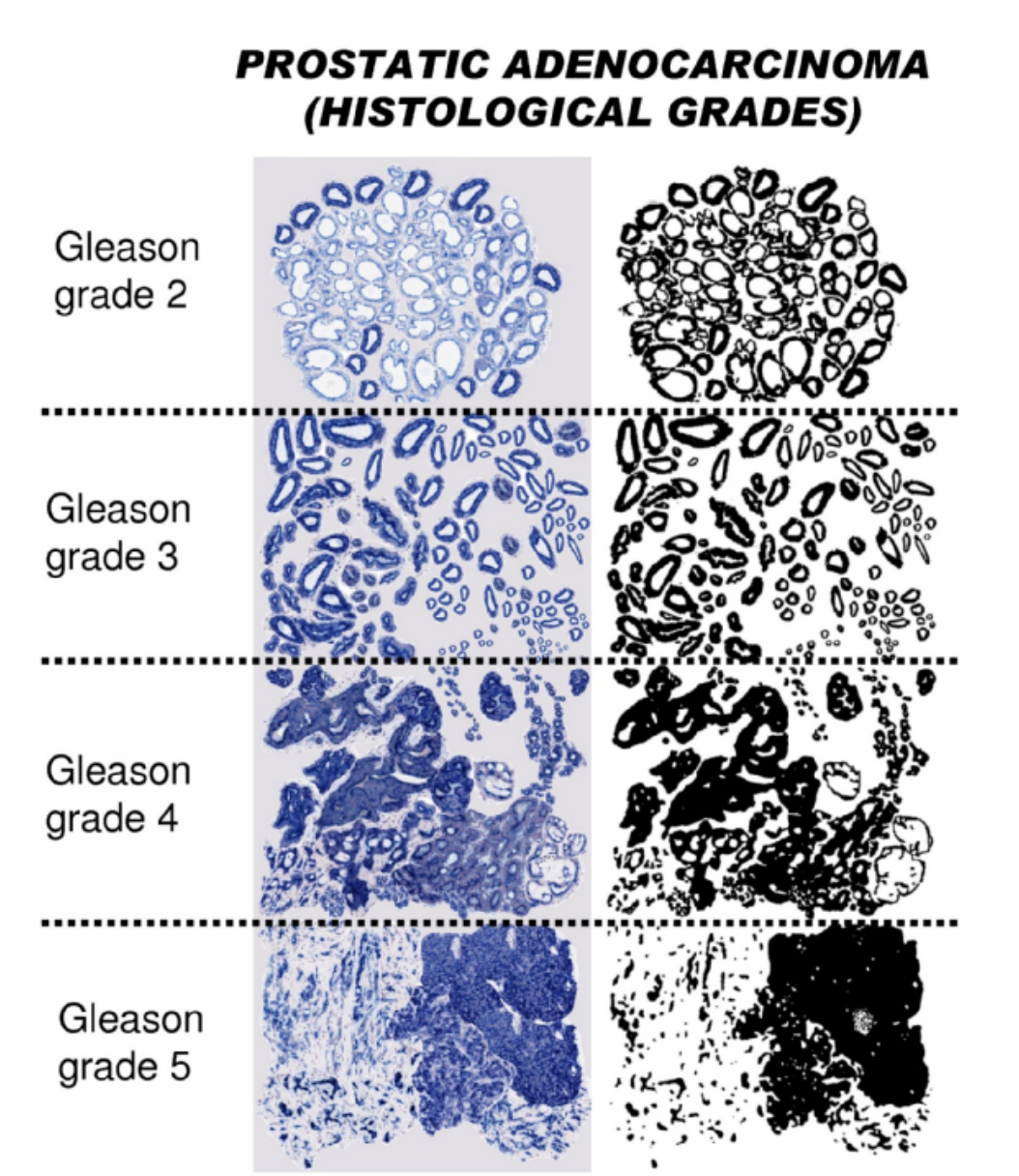


Figure 13. Updated Gleason patterns. Left: true prostate cancer patterns according to Gleason grades. Patterns were cut from digital images of 2IHC slides. Right: our proposal for a new reference picture of modified Gleason scores.

# CONCLUSIONS

The key findings of the study contribute to the existing literature regarding prostate cancer development, diagnostics of small cancers, and prognostic factors in the PSA era.

The main conclusions of this study are as follows.

1. BAX and BCL-2 are overexpressed in some foci in morphologically normal areas of cancerous prostates, consistent with the field effect theory.
2. Routine 2IHC of interval sections of prostate needle biopsies improves diagnostic sensitivity of prostate cancer with less time spent on microscopy.
3. Gleason score, PSA value, multiple PNI and cT-stage are independent prognostic factors in endocrine-treated prostate cancer patients. Gleason score 3+4 versus 4+3 is a prognostic watershed between low-grade and high-grade cancer.
4. All biomarkers analyzed (EZH2, Ki-67 and MCM7) are significant individual prognosticators, and have comparable relative risks to those of histopathological variables. Ki-67 is especially useful in assessing the risk of a subgroup of patients with Gleason scores of 7.
5. Worst and overall Gleason scores provide comparable information in the modified Gleason score era. The use of pooled biopsies is a cost-efficient way to perform routine 2IHC, and its use should be weighed against the missed locus information.

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# ORIGINAL COMMUNICATIONS

BJUI
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# Histopathological variables and biomarkers enhancer of zeste homologue 2, Ki-67 and minichromosome maintenance protein 7 as prognosticators in primarily endocrine-treated prostate cancer

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Study Type – Prognosis (case series)
Level of Evidence 4

**What's known on the subject? and What does the study add?**
Gleason score is a strong prognostic factor, but its reproducibility is not optimal. Our data show that multipte perineural invasion and Ki-67 index are signs of early biochemical progression in patients treated with hormonal therapy.

## OBJECTIVE

- To evaluate the prognostic value of histopathological variables and immunostainings of biomarkers enhancer of zeste homologue 2 (EZH2), Ki-67 and minichromosome maintenance protein 7 (MCM7) from core biopsies of hormonally treated patients with prostate cancer.

## PATIENTS AND METHODS

- Biopsies of 247 primarily endocrine-treated patients were analysed for histopathological characteristics and Gleason scores (GS) according to the revised guidelines of International Society of Urologic Pathology (ISUP) consensus conference 2005.
- Immunohistochemical stainings were analysed with the aid of digital image analysis.
- The prognostic value of the histopathological variables and the biomarkers was analysed with univariate and multivariate Cox regression analysis, with biochemical recurrence as an endpoint.

## RESULTS

- Biomarkers EZH2 (relative risk [RR] 2.0, 95% confidence interval 1.2–3.3), Ki-67 (3.4, 2.1–5.5) and MCM7 (2.4, 1.5–3.9) were significantly associated with progression-free survival in a univariate analysis.
- Ki-67 immunostaining index detected high-risk patients with GS of 7 (9.1, 8.0–10.3).
- In a multivariate analysis with non-conventional GS groups 5–7 (3 + 4), 7(4 + 3)–8, and 9–10, the independent prognostic markers were pretreatment GS (2.2, 1.5–3.2), prostate-specific antigen (PSA) level (2.1, 1.1–4.2), perineural invasion (PNI) (1.6, 1.2–2.2), and clinical T-stage (cT) (1.9, 1.0–3.7).
- Combination of the independent markers (PSA level >20 ng/mL or GS >3 + 4 or PNI >3 or cT >2) yielded best risk stratification (RR 11.6, 10.4–12.7).

## CONCLUSIONS

- GS remains one of the most important prognostic factors in prostate cancer. However, the refined guidelines by ISUP 2005 might have shifted the threshold between low-grade and high-grade cancers from GS 6 vs 7 to GS 3 + 4 vs 4 + 3.
- PNI is an independent prognostic marker superior to cT.
- Ki-67 is the most useful biomarker in detecting patients with GS = 7 at high risk for progression.

## KEYWORDS

prostate cancer, androgen deprivation, Gleason score, perineural invasion, Ki-67, ISUP 2005

## INTRODUCTION

Prostatic adenocarcinoma is the most frequent non-cutaneous malignancy in the Western world and causes substantial mortality and morbidity [1]. Treatment options in localized disease include radical nerve-sparing prostatectomy, radiation therapy and active surveillance [2]. Androgen deprivation therapy (ADT) is recommended for extraprostatic or metastatic disease [2]. Primary ADT has also been used in localized

disease, despite a lack of conclusive evidence for its benefit [3]. Almost all patients initially respond to androgen deprivation. However, disease progression eventually occurs due to the emergence of castration-resistant prostate cancer (CRPC) [4]. For such CRPC, there are no curative therapies available, although recent trials have indicated that docetaxel prolongs the life of patients with CRPC for a few months [5].

Several nomograms have been created to predict the outcome. The strongest prognostic factors are clinical TNM stage, pretreatment PSA level, and Gleason score (GS) [6,7]. Other suggested prognostic factors are patient age, the total percentage of cancer (TPC), the greatest percentage of a biopsy core involved by cancer (GPC), the percentage of cancer-positive cores (CPC) and perineural invasion (PNI) [8–13]. The prognostic value of these variables has not been evaluated systematically from biopsy specimens of primarily ADT-treated patients. Several immunohistochemical and genetic markers of outcome have also been proposed. However, none of them has become routinely used in clinical practice. Most of the biomarkers are associated with cell proliferation activity and cell cycle control. One of the most promising and extensively studied prognostic proliferation markers is Ki-67 (MIB-1) [14–18]. Other potential cell proliferation biomarkers include enhancer of zeste homologue 2 (EZH2), which is an evolutionary highly conserved epigenetic regulator that functions as a histone methyltransferase [19]. Overexpression of EZH2 is associated with poor prognosis in several cancers, including prostate carcinoma [20–24]. Our previous study indicated that overexpression of EZH2 is an independent prognostic factor in surgically treated patients [25]. Another suggested prognostic marker is minichromosome maintenance protein 7 (MCM7), which is a critical component of the DNA replication licensing complex [26,27]. In our earlier study of prostatectomy-treated patients, MCM7 was a strong prognostic marker, especially in combination with EZH2 [25].

Most of the recent prognostic studies have focused on patients treated with prostatectomy. However, many patients are, even today, primarily treated with hormonal therapy. The utility of docetaxel in treating prostate cancer has raised questions about how to identify patients who will progress early during endocrine treatment. The aim of the present study was to evaluate the prognostic significance of the suggested tissue markers in endocrine-treated patients with prostate cancer.

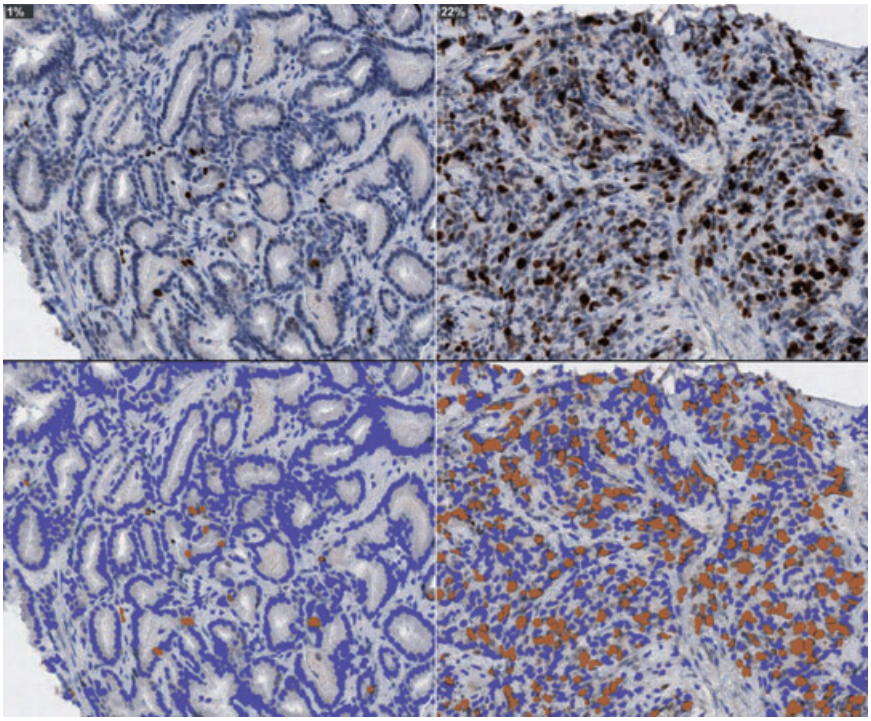
## MATERIAL AND METHODS

The present study has been approved by the Ethical Committee of Tampere University Hospital and the National Authority for Medicolegal Affairs. From 1999 to 2003, 295 consecutive new patients with prostate cancer diagnosed from core biopsies were primarily hormonally treated at Tampere University Hospital. This is approximately 25% of all the prostate cancers treated at the hospital. The indication for endocrine treatment was advanced disease or patients being unwilling or unsuitable for therapy of curative intent due to their general condition but wanting to have active treatment instead of watchful waiting. None of the patients received other forms of cancer treatment, such as radiation or surgery, before progression. Representative formalin-fixed and paraffin-embedded first cancer diagnosis biopsy samples were available from 247 (83.7%) patients. Complete clinical follow-up data were available from 292 patients. Biochemical progression was defined as a ≥25% rise in PSA level with a PSA value ≥2.0 ng/mL above nadir in two consecutive measurements according to The Prostate Cancer Clinical Trials Working Group guidelines [28]. To assess the M-stage of the patients, bone scintigraphy was done in all symptomatic patients and in asymptomatic patients when PSA level was ≥20 ng/mL or when the prostate cancer was histologically aggressive (original GS = 8–10). In the analyses, MX patients were considered as M0 due to the fact that they represented cases with low PSA levels and low GS and because their prognosis was as good as the prognosis for M0 patients. The distribution of primary hormonal treatments was surgical castration ($n = 55$), luteinizing-hormone releasing-hormone analogue ($n = 208$), antiandrogen bicalutamide ($n = 27$) and maximal androgen blockade ($n = 3$).

One slide from each prostate lobe was available. The mean (range) number of core biopsies from one lobe was 4.5 (1–7). The most representative haematoxylin and eosin (H&E)-stained slide, consisting of biopsies from the left or right lobe, was selected and scanned with Aperio ScanScope® XT (software version 9; Aperio Technologies, Vista, CA, USA) and viewed in JPEG2000 format using JVSview software (version 1.2) [29]. The re-evaluated variables were GS, GPC, CPC, number of nerves with PNI, and the diameter of the greatest perineurally invaded nerve. The GS were re-evaluated according to the new recommendations by International Society of Urologic Pathology (ISUP) [30]. The combined GS for the re-evaluated biopsies of one lobe was used in the analyses. For GPC, the percentage of cancer in each core was estimated as the proportionate length of cancer to the total length of the biopsy. The length of benign tissue between separate expansive cancer foci was subtracted. The length of infiltrating carcinoma glands intervening normal glands was counted as carcinoma. TPC (including both lobes) and WHO grades were obtained from the original pathology reports. PNI was defined as infiltration of carcinoma into the perineural space. An entire encircling was not required. The diameter of greatest nerve with PNI was measured from virtual slide screenshots using the public domain image analysis software ImageJ (version 1.36b) (http://rsbweb.nih.gov/ij/index.html) [31]. The evaluations were done by one of the authors (TTT) in a blinded fashion, i.e. with no awareness of the clinical outcome.

Immunostainings were performed with antibodies against Ki-67 (MM1, Novocastra™ Laboratories Ltd., Newcastle Upon Tyne, UK), EZH2 (NCL-L-EZH2, clone 6A1, Novocastra™ Laboratories Ltd), and MCM7 (sc-9966, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) with Power Vision1™ Poly-HRP Histostaining Kit (ImmunoVision Technologies Co, Daly City, CA, USA) according to the manufacturers' instructions. The stainings were carried out in Autostainer 480 (Lab Vision Corp, Fremont, CA, USA). Briefly, slides were autoclaved in pretreatment buffer (5 mM Tris-HCl/L mM EDTA, pH 9) at 121 °C for 2 min, followed by incubation with the primary antibody diluted in pre-block solution (Ki-67 1:1500, EZH2 1:300, MCM7 1:500) overnight. After washing and blocking, the bound primary antibody was visualized with the PowerVision™ Poly-HRP IHC Detection Kit (ImmunoVision Technologies Corporation, Brisbane, CA, USA). The slides were counterstained with haematoxylin. The digitalization of the immunostained slides was performed using the virtual slide scanner set-up as described earlier. Screenshots of the three hotspot areas showing the highest immunostaining were

*FIG. 1. An example of a typical automated analysis of two Ki-67 immunostainings. Top panels: hotspot areas were selected and captured for analysis of the immunostainings (DAB brown and haematoxylin blue); bottom panels: the result of automated analysis of the same hotspots (below). Magnification, ~100×.*




captured from each slide. The images were analysed with ImmunoRatio, which is a tool for analysing nuclear immunostainings in haematoxylin-counterstained tissue sections [32]. Analysis was based on the colour deconvolution for the separation of the staining components [diaminobenzoate (DAB) brown and haematoxylin blue] and adaptive thresholding for defining staining positivity [33]. The proportion of brown-stained area over brown + blue was defined as the labelling index. The results of the automated analysis were verified by one of the authors (TTT) comparing the original image to the segmented image (Fig. 1).

For statistical analysis, Fisher's exact, chi-square and one-way ANOVA tests were used to evaluate the associations between the variables. Survival analysis was performed using the Kaplan–Meier method and the statistical significance of survival differences between patient groups was determined with the Mantel–Cox test. The univariate and multivariate Cox regression analyses were performed to calculate the relative risk (RR) estimates and to evaluate the independence of the prognostic markers.

## RESULTS

The median (range) age of the patients at the time of diagnosis was 73.6 (52.7–88.8) years. The median (range) follow-up time was 56.5 (8–104) months. The months pretreatment PSA level was 16.5 (1.9–10 750) ng/mL. Over half (54.9%, 162/295) of the patients had localized disease (cT1–T2). One-third of the patients (33.6%) experienced PSA progression. Cancer-specific mortality was 6.4% (19/295) and deaths due to other reasons occurred in 25.7% (76/295). Re-evaluated GS and other histopathological variables were successful in 247 cases. GS distribution was as follows: GS < 7, $n = 60$ (24.3%); GS = 7, $n = 79$ (32.0%); and GS > 7, $n = 108$ (43.7%). There were no cases with GS = 2–4. The distribution of the basic characteristics is presented in Table 1.

Because the tissue in the paraffin blocks runs out after repeated sectioning, the number of successful cases varied in different stainings. Immunohistochemical analysis was successful in 216 cases for Ki-67, 209 cases for EZH2, and 211 cases for MCM7. For the data analysis, mean values from the three hotspots were used. Two example cases including H&E stainings and immunostainings of the needle biopsies can be viewed at http://jvsmicroscope.uta.fi/tolpub2010/.

*TABLE 1 Distribution of the clinicopathological variables*

| | *n* (%) |
|---|---|
| Clinical T-stage (*n* = 295) | |
| cT1–2 | 161 (55) |
| cT3–4 | 134 (45) |
| M-stage (*n* = 295) | |
| Mx* | 127 (43) |
| M0 | 111 (38) |
| M1 | 57 (19) |
| Gleason score (*n* = 247) | |
| 2–4 | 0 (0) |
| 5 | 4 (2) |
| 6 | 56 (23) |
| 7† | 79 (32) |
| 8 | 42 (17) |
| 9 | 53 (21) |
| 10 | 13 (5) |
| PSA level, ng/mL (*n* = 295) | |
| ≤20 | 168 (57) |
| >20 | 127 (43) |
| Age, years (*n* = 295) | |
| <60 | 13 (4) |
| 60–70 | 70 (24) |
| 70–80 | 174 (59) |
| 80+ | 38 (13) |

**Mx were considered M0 in statistical calculations. †n = 50 for GS 3 + 4 and n = 29 for GS 4 + 3.*

The Kaplan–Meier curves of the progression-free survival are shown in Figs 2–4. In the subgroup of GS 7, the rate of progression was lower in Gleason grades 3 + 4 than in 4 + 3. The best prognostic value was achieved by reclassifying GS grouping as 5–7 (3 + 4), 7 (4 + 3)–8, and 9–10 ($P < 0.001$, log-rank test) (Fig. 2A). Also, the traditional GS groups < 7, 7 and >7, were strongly associated with progression-free survival ($P < 0.001$) (Fig. 2B). Both the advanced against localized (T3–4 against T1-2) cT-stage ($P < 0.001$) and M-stage (M1 against M0) ($P < 0.001$) were significantly associated with progression-free time (Fig. 2C,D). In the analysis of pretreatment PSA level, we used a dichotomized grouping of PSA ≤ 20, >20 ng/mL as reported by Graff *et al.* [34]. PSA level

was found to be highly significantly associated with progression-free survival ($P < 0.001$) (Fig. 2E). The other histopathological features, CPC ($P < 0.001$, Fig. 2F), GPC ($P < 0.001$, Fig. 2G), TPC ($P < 0.001$, Fig. 2H) and the number of PNI ($P < 0.001$, Fig. 3A), were also associated with progression-free survival. WHO grade, which was derived from the original pathology reports, showed a significant association with progression ($P < 0.001$, Fig. 3B). The only histopathological variable not associated with progression was the diameter of the greatest nerve with PNI ($P = 0.75$).

Ki-67 staining index over 10% identified a quintile with a very high risk of progression ($P < 0.001$, Fig. 3C). For the MCM7 ($P = 0.001$) and EZH2 ($P = 0.0041$) immunostainings, the best dichotomous threshold values were determined with Kaplan–Meier curves utilizing Mantel–Cox tests, both of which gave values close to the medians (Fig. 3D,E, respectively).

Inside the sub-group of patients with GS = 7, the progression-free time for patients with Gleason grading 4 + 3 was significantly shorter than for patients with grading 3 + 4 ($P = 0.013$, Fig. 4A). In the same patient group, Ki-67 expression with a threshold value of 10% was highly capable of finding patients with early PSA recurrence ($P < 0.001$, Fig. 4B).

The RR and 95% CIs were first calculated with univariate analysis utilizing a Cox regression model and are presented in Table 2. All of the markers were statistically significant as individual prognostic factors. To evaluate association of the variables against each other, we utilized a chi-squares test of the categorized markers. All variables were significantly ($P < 0.05$) associated with each other. Thus, to evaluate the independent prognostic power of the histopathological variables and the biomarkers, we utilized a multivariate Cox regression model. The first multivariate analysis was performed with the conventional GS grouping of <7, 7 and >7. Pretreatment PSA level, GS, number of PNI and cT-stage were found to be independent predictors of progression (Table 2). None of the proliferation biomarkers showed independent prognostic power. Because GS groups 3 + 4 and 4 + 3 showed a clearly distinct prognosis (Fig. 2F), we performed a second multivariate analysis with reclassified GS groupings as 5–7 (3 + 4), 7 (4 + 3)–8, 9–10. In the analysis, the modified GS grouping was the strongest independent prognostic factor. Other independent prognosticators were pretreatment PSA level, number of PNI and cT-stage (Table 2).

Finally, we combined the four independent markers from the multivariate analysis into a single risk estimate of progression. The threshold values were PSA level >20 ng/mL, cT-stage >2, and PNI >3. In addition, three cut-offs for Gleason scores were tested (Fig. 4C–E). The best risk stratification (RR = 11.6, 95% confidence interral 10.4–12.7) was achieved when Gleason score threshold 3 + 4

*FIG. 2. Kaplan–Meier progression-free survival curves according to reclassified GS (**A**), GS (**B**), cT-stage (**C**), M-stage (**D**), pretreatment PSA-value (**E**), percentage of cancer-positive cores (CPC) (**F**), greatest percentage cancer in a single biopsy (GPC) (**G**), and total percentage cancer (TPC) (**H**). P values according to Mantel-Cox test are shown.*

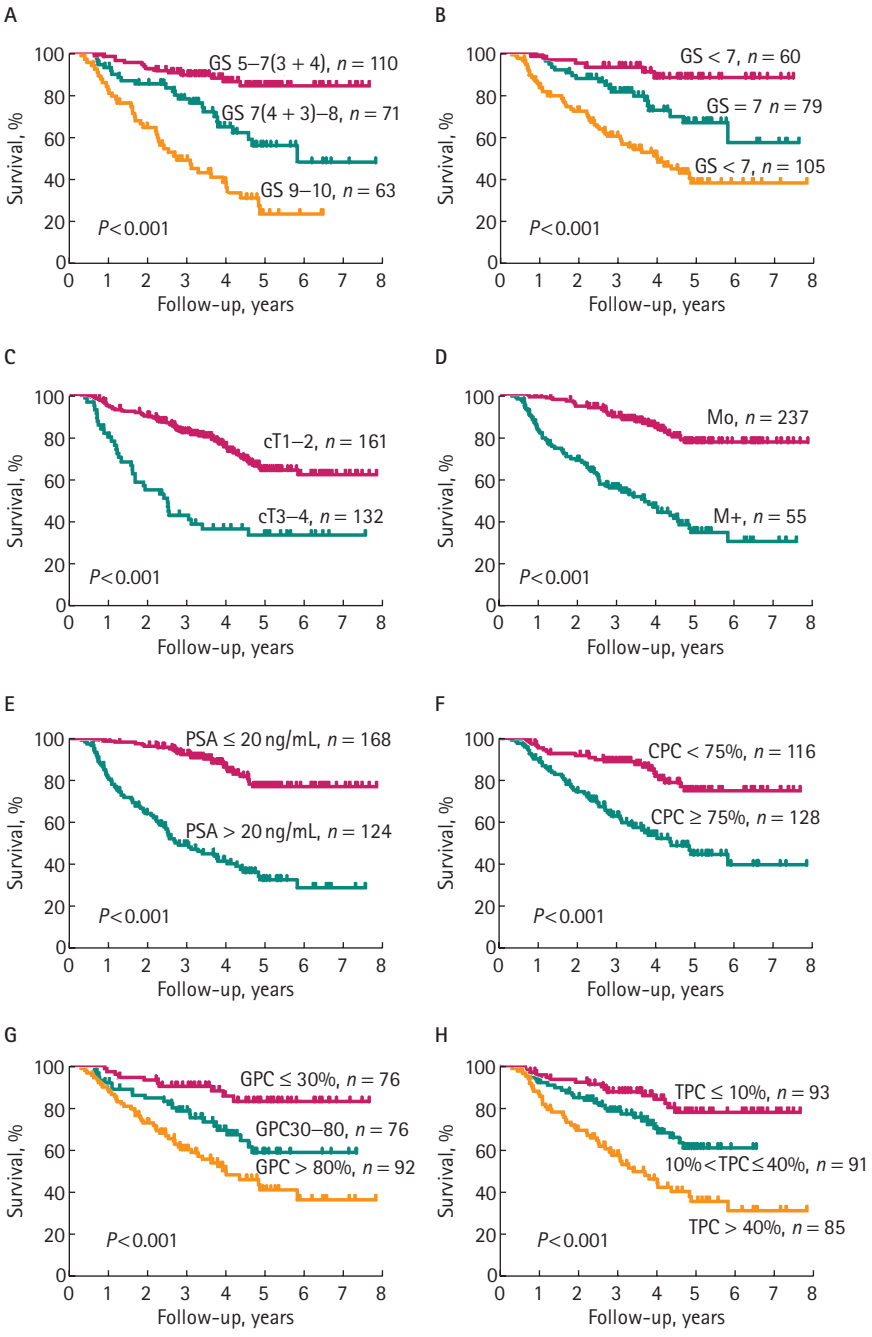
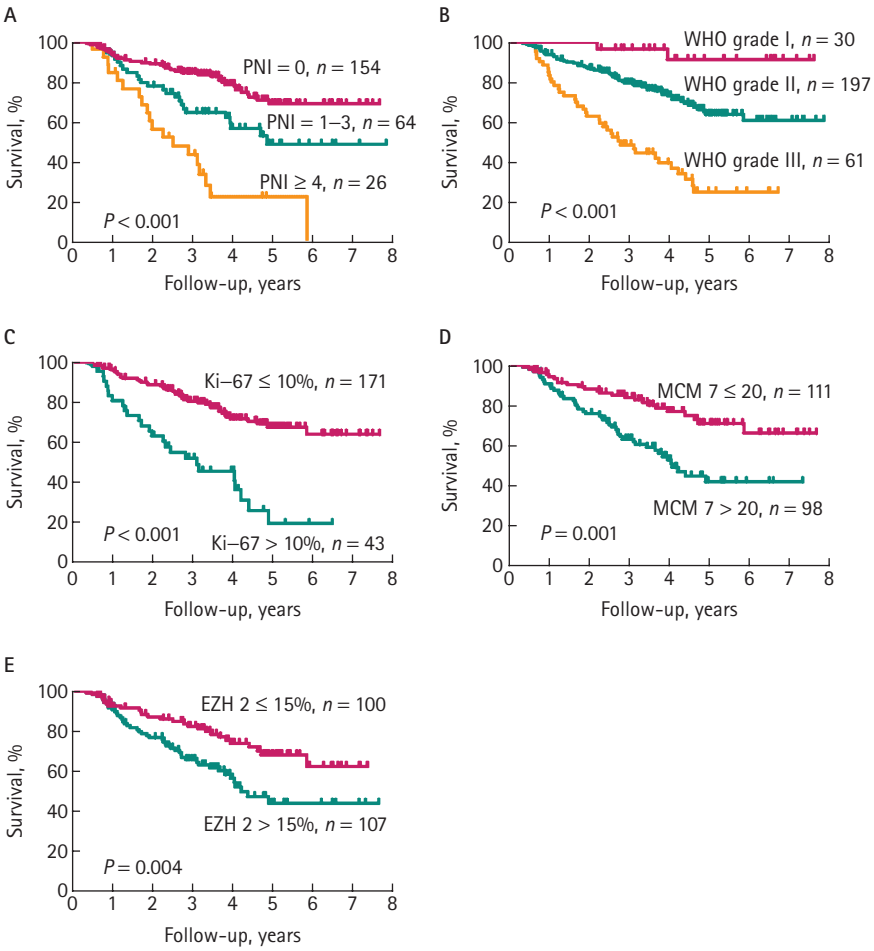


*FIG. 3. Kaplan–Meier progression-free survival curves according to PNI (**A**), WHO grade (**B**), Ki-67 (**C**), MCM7 (**D**), and EZH2 (**E**). P values according to Mantel–Cox test are shown.*




was utilized (Fig. 4C). Risk for progression was very low (four of 61, 6.6%) in patients in the low-risk arm.

## DISCUSSION

Primary ADT is offered to patients with advanced disease or those who are otherwise not suitable for intent-to-cure therapy, e.g. elderly patients with localized disease [2,3]. In patients with locally advanced tumours and patients with poorly differentiated localized cancers, the use of ADT is associated with improved cancer-specific survival [3]. However, a majority of the patients with a well- to moderately differentiated localized disease are not likely to benefit from ADT [3]. Graff *et al.* [34] have shown that in patients with localized cancer receiving primary ADT, GS ≥7, PSA level ≥20 ng/mL and a low comorbidity index are independent predictors of shorter cancer-specific survival. After progression to CRPC, the significance of GS and PSA level diminishes, and the most important prognostic factors are general markers, such as haemoglobin, lactate dehydrogenase and albumin [35]. Since docetaxel has now been shown to prolong the lives of patients with CRPC [5], the question of timing of the treatments has arisen. There is a need for indicators for the early use of docetaxel [5]. Until recently, there have not been, for example, nomograms for endocrine-treated non-CRPC patients. In addition, most studies with detailed histopathological interpretations of biopsy cores have concentrated on radical treatments. Recently, a risk assessment tool for patients receiving primary ADT was developed in a large multicentre study [36]. Like other nomograms, the tool Japan Cancer of the Prostate Risk Assessment (J-CAPRA) is based on PSA level, GS and cTNM-stage and validated in both localized and advanced disease. The major limitation of the model is that it lacks complementary histopathological information from biopsy cores.

In the present study, almost all histopathological variables (except diameter of the greatest nerve invaded) were strong indicators of progression-free survival. The modified GS was the strongest independent prognostic factor. Patients with a GS of 5–7 (3 + 4) had a very low risk for progression. Patients with a GS of 4 + 3 had a moderate risk, which was similar to that of patients with GS 8. The risk for progression in patients with a GS of 9–10 was very high, and also significantly higher than in patients with GS 7 (4 + 3)–8. It has previously been shown that GS 4 + 3 cancers behave more aggressively than GS 3 + 4 cancers in surgically treated patients [37–39]. Here, our data suggest that GS 3 + 4 vs 4 + 3 is a critical prognostic threshold between very low and moderate risk in patients treated with ADT. Also, GS 9–10 cancers behave clearly more aggressively than those of GS 8, and these cancers should not be grouped together.

Gleason score is assessed by a pathologist on the basis of glandular architecture. Reproducibility of GS is not optimal due to extreme variation of the architectural patterns and because the interobserver variability has been rather high [40,41]. The refinement of the Gleason grading system in needle biopsies by the ISUP [30] has proven to be valuable in identifying more patients with aggressive disease [42,43] and in improving interobserver agreement [44]. However, the comparison of the results of different studies over time has been difficult because of the changing definitions of GS [45]. Previous studies on radical prostatectomy specimens have suggested tumours with modified GS 3 + 4 might behave in a similar way to GS 3 + 3 tumours, whereas GS 4 + 3 tumours are more aggressive [46]. Refined guidelines of the ISUP consensus conference 2005 may have shifted the traditional low-grade vs high-grade threshold, but there has been a lack of studies showing this on core biopsy material [44]. The results on needle biopsies from the present study support earlier findings on prostatectomy specimens by demonstrating that patients with GS 4 + 3 have poorer prognosis than patients with GS 3 + 4.

The risk ratio for WHO grade was high in univariate analysis, but it did not reach a statistically independent prognostic value in the multivariate analysis. When a three-step

classification system is in use, pathologists tend to classify most cases as intermediate. Here, over two-thirds of cases were considered as WHO grade II, which is a severe limitation of its usefulness for clinicians.

Pathological stage is the most important predictor of outcome and a golden standard in measuring local spread and tumour volume. In patients not treated with prostatectomy, there are several ways to estimate the pT-stage and/or tumour volume, including cT-stage, CPC [11,47], GPC [8] and TPC [12]. Indeed, all these variables show strong associations with each other. In the univariate analysis, CPC had the highest RR of the tumour volume estimates. Also, both GPC and TPC showed significant prognostic value. CPC, GPC and TPC provide important complementary data to the clinical T-stage. The reproducibility of percentile estimation of GPC and TPC should be optimized with the aid of digital image analysis in future.

Perineural invasion was found to be an independent prognostic marker in both multivariate analyses. Previously, the findings on the significance of PNI have been controversial. The presence of PNI in needle biopsies has shown independent predictive value of aggressive phenotype and Gleason upgrading in prostatectomy specimens [13,48]. However, PNI has lacked independent prognostic power in most studies on prostatectomy patients [8,13,49]. In one study on patients treated with external beam radiotherapy, PNI predicted biochemical progression [50]. The increasing diameter of the largest PNI has been associated with a higher rate of biochemical progression in surgically treated patients [51]. PNI is commonly seen in radical prostatectomy specimens close to the capsule or in extraprostatic tissue. Therefore we assumed that multiple PNI would correlate with the extent of capsular invasion. In the present study, not only the mere presence of PNI but also the number of perineurally invaded nerves was significantly associated with progression-free time. These data show that the presence of multiple PNI is an independent prognostic marker in endocrine-treated patients, and it should be included in the pathology report on needle biopsies.

It was indicated more than a decade ago that cell proliferation activity is a prognostic marker in endocrine-treated prostate cancer [52]. Also, a high Ki-67 labelling index was shown to be an independent prognostic factor in some studies [53]. In the present study, Ki-67 was a statistically significant prognostic marker in univariate analysis but not an independent marker in multivariate analyses; however, patients with Ki-67 >10% were at a very high risk of progression (Fig. 3C). In patients with a GS of 7, reproducibility of the GS is critical. A Ki-67 labelling index >10% was significantly associated with poor prognosis in patients with a GS of 7 and could be helpful in their risk assessment (Fig. 4B).

Over-expression of both MCM7 and EZH2 has been found in cancers with aggressive features, but it has not been thoroughly studied early in patients receiving primary ADT. Both biomarkers were significant prognostic markers in univariate analysis, but they lacked independent prognostic power in multivariate analysis. The RR values (95% CI) for the three biomarkers were 3.4 (2.1–5.5) for Ki-67, 2.4 (1.5–3.9) for MCM7, and 2.0 (1.2–3.3) for EZH2. These values are similar to the currently used histopathological variables included in pretreatment nomograms. The intensity of immunostaining is variable, and often, the objective discrimination between positive and negative nuclei is difficult. Computer-based automated image analysis is a useful approach, as proved by the analysis of the immunostainings in the present study. Due to variables used in the programming, the

*FIG. 4. Kaplan–Meier progression-free survival curves in GS 7 cases according to scores 4 + 3 and 3 + 4 (**A**), and Ki-67% (threshold 10%) (**B**), as well as in all patients stratified for low- and high-risk groups according to the following criteria for high-risk group GS > 3 + 4 or PSA level > 20 ng/mL or cT-stage > 2 or PNI > 3 (**C**), GS > 7 or PSA level > 20 ng/mL or cT-stage > 2 or PNI > 3 (**D**), and GS > 8 or PSA level > 20 ng/mL or cT-stage > 2 or PNI > 3 (**E**). RR values and 95% CIs according to Cox univariate analysis and P values according to Mantel–Cox test are shown.*

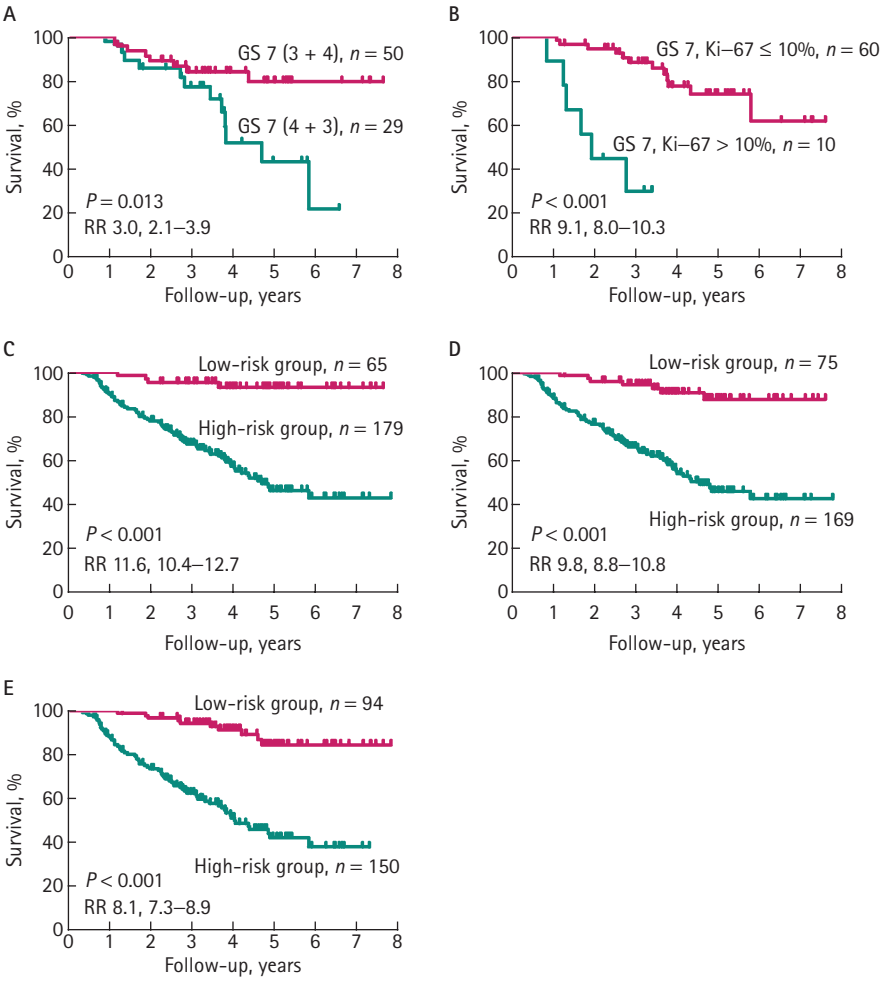


*TABLE 2 Univariate and multivariate analyses of the prognostic markers*

| | Univariate | | 1° multivariate | | 2° multivariate | |
|---|---|---|---|---|---|---|
| Parameter* | RR (95% CI) | *P* value | RR (95% CI) | *P* value | RR (95% CI) | *P* value |
| PSA level, ng/mL | 5.6 (3.6–8.7) | <0.001 | 2.6 (1.3–4.9) | <0.001 | 2.1 (1.1–4.2) | <0.0001 |
| cT-stage | 4.6 (3.0–7.2) | <0.001 | 2.0 (1.1–3.9) | 0.031 | 1.9 (1.0–3.7) | 0.057 |
| WHO grade | 3.3 (2.3–4.8) | <0.001 | NS | | NS | |
| Metastasis | 3.2 (2.1–4.9) | <0.001 | NS | | NS | |
| Ki-67 IHC | 3.4 (2.1–5.5) | <0.001 | NS | | NS | |
| Reclassified GS | 2.6 (2.0–3.6) | <0.001 | Not included in the analysis | | 2.2 (1.5–3.2) | <0.001 |
| CPC | 3.2 (1.9–5.2) | <0.001 | N.S. | | NS | |
| GS | 2.6 (1.8–3.6) | <0.001 | 2.1 (1.4–3.2) | <0.001 | Not included in the analysis | |
| PNI | 2.2 (1.7–3.0) | <0.001 | 1.6 (1.2–2.2) | 0.002 | 1.6 (1.2–2.2) | 0.003 |
| TPC | 2.1 (1.6–2.8) | <0.001 | NS | | NS | |
| GPC | 2.1 (1.5–2.8) | <0.001 | NS | | NS | |
| MCM7 IHC | 2.4 (1.5–3.9) | <0.001 | NS | | NS | |
| EZH2 IHC | 2.0 (1.2–3.3) | 0.004 | NS | | NS | |

**Threshold values: PSA level ≤20, >20 ng/mL; cT-stage, cT3–4 against cT1–2; WHO grade, I, II, III; metastasis, M+ against M0; Ki-67, ≤5%, >5–10%, >10%; reclassified GS, 5–7 (3 + 4), 7(4 + 3)–8, 9–10; CPC, <75%, 75–100%; GS, <7, 7, >7; PNI, 0, 1–3, >3; TPC, ≤10%, >10–40%, >40%; GPC, ≤30%, >30–80%, >80%; MCM7, ≤20%, >20%; EZH2, ≤15%, >15%.*

results of automated segmentation are not strictly the same as manual counting, but they are very similar. The major advantage of using this method is high reproducibility in detecting immunostained nuclei and speed of counting hundreds of cells.

The prognosis of prostate cancer in elderly men is good, in general, and cancer-specific mortality is significantly lower than deaths due to other reasons [54]. Because of that, we were unable to use cancer-specific death as the primary endpoint. However, PSA progression is believed to be a reliable surrogate marker of survival [28]. Another weakness of the present study is that, for large fraction of patients, bone scintigraphy was not performed, although the probability that these patients would have had bone metastases is very low [55]. The third weakness is related to needle biopsy material: the evaluation of the histopathological variables was based on the original serial-cut H&E slides with more complete cores, whereas immunohistochemistry was performed on sections from the block remnants, which could cause a bias under-estimating the significance of the immunostainings. The original slides or paraffin blocks were not available from 45 patients. As there were no statistical differences between the missed cases and the studied cases, we consider the material to be unbiased.

In conclusion, there are many significant prognostic factors in prostate cancer. There were four common independent factors in both multivariate analyses: GS, PSA level, PNI and cT-stage. Combination of these variables provides a strong prognosticator with a RR of more than 10. As all of the studied variables were associated with each other, the independent prognostic value of the biomarkers is dependent on the accuracy of the subjective GS. Although GS was superior to the studied biomarkers in multivariate analysis, its reproducibility is not optimal, and the scores are variable between individual pathologists and clinics. We believe the use of Ki-67 with a threshold value of 10% in clinical routine could support clinical decision-making. It could be especially helpful in determining the risk and choice of treatment for patients with a GS of 7.

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## CONFLICT OF INTEREST

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Abbreviations: **ADT**, androgen deprivation therapy; **CPC**, percentage of cancer-positive cores; **CRPC**, castration-resistant prostate cancer; **DAB**, diaminobenzoate; **EZH2**, enhancer of zeste homolog 2; **GPC**, greatest percentage of a biopsy core involved by cancer; **GS**, Gleason score; **ISUP**, International Society of Urologic Pathology; **MCM7**, minichromosome maintenance protein 7; **PNI**, perineural invasion; **RR**, relative risk; **TPC**, total percentage of cancer.

# Overall and worst Gleason scores are equally good predictors of prostate cancer progression

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# Abstract

## Background

Prostatic needle biopsies are individually paraffin-embedded in 57 % of the European pathology laboratories, whereas the rest of laboratories embed multiple (2 - 6) biopsies per one paraffin-block. Differences in the processing method can have a far-reaching effect, because reporting of the Gleason score (GS) is different for individually embedded and pooled biopsies, and GS is one of the most important factors when selecting treatment for patients. Also, Gleason scoring has experienced several modifications during the past decade. So far, only one study has compared the prognostic abilities of worst (WGS) and overall (OGS) modified Gleason scores after the ISUP 2005 conference.

## Methods

The study material consisted of needle biopsies from 236 prostate cancer patients that were endocrine-treated in 1999-2003. Biopsies from left side and right side were embedded separately. Haematoxylin-eosin-stained slides were scanned and analyzed on web-based virtual microscopy. Worst and overall Gleason scores were assessed according to the modified Gleason score schema after analyzing each biopsy separately. The compound Gleason scores (CGS) were obtained from the original pathology reports. The prognostic ability of the three scoring methods to predict biochemical progression was compared with Kaplan-Meier survival analysis and univariate and multivariate Cox regression analyses.

## Results

The median follow-up time of the patients was 64.5 months (range 0-118). The modified GS criteria led to upgrading of the Gleason sums compared to the original CGS from the pathology reports 1999-2003 (mean 7.0 for CGS, 7.5 for OGS, 7.6 for WGS). In 43 cases WGS was > OGS. In a univariate analysis the relative risks were 2.1 (95%-confidence interval 1.8-2.4) for CGS, 2.5 (2.1-

2.8) for OGS, and 2.6 (2.2-2.9) for WGS. In a multivariate analysis, OGS was the only independent prognostic factor.

### Conclusions

All of the three Gleason scoring methods are strong predictors of biochemical recurrence. The use of modified Gleason scoring leads to upgrading of GS, but also improves the prognostic value of the scoring. No significant prognostic differences between OGS and WGS could be shown, which may relate to the apparent narrowing of the GS scale from 2-10 to 5-10 due to the recent modifications.

## Background

Grading of prostatic needle biopsies has experienced several refinements in the last decade. First, Epstein suggested that a diagnosis of Gleason score (GS) 2+2=4 cancer should not be made on the needle biopsies, because subsequent radical specimen showed upgrading in virtually all cases [1]. Next, worst Gleason score (WGS) was shown superior to overall Gleason score (OGS) in predicting the final GS of the radical specimen, yielding fewer cases of unwanted upgrading events [2]. Third major adaptation was made in the consensus conference of International Society of Urological Pathology 2005, leading to a refinement called modified GS [3]. In that, any aggressive cancer seen on the needle biopsies should be recorded and incorporated to the GS, even if present in small amount.

Worst Gleason score (WGS) is recommended for individually processed biopsies by ISUP 2005 consensus conference [3]. In the case of pooled biopsies, the exact number of biopsies is sometimes difficult to know due to tissue fragmentation and/or overlapping of the biopsies, and thus, WGS cannot be reliably assessed [3].

According to a recent survey among European pathology laboratories, approximately one half of the participants use individually processed biopsies, while the others immerse multiple biopsies per formalin container without special identification tags (Lars Egevad, personal communication). Individually processed biopsies allow clinicians to localize the histopathological findings to the anatomic biopsy site. In addition, when the biopsy cores are individually embedded in paraffin blocks, a separate GS can be assessed for each biopsy, and the worst of them is usually reported to the clinicians to guide the treatment. Instead, the uropathologists did not reach consensus whether to use worst or overall GS in the case when multiple cancer-containing biopsies are pooled to one formalin container without identification tags [3].

A few studies comparing OGS and WGS have been published and only one of them after the ISUP conference [4]. In three studies WGS at any biopsy site was better than OGS at predicting the pathological T-stage and GS in radical prostatovesiculectomy specimens [2, 4, 5] whereas in one study, OGS performed better in predicting progression-free survival in patients treated with radiotherapy [6].

Our earlier study analyzing biopsies from endocrine-treated patients indicated that OGS was the strongest independent prognosticator of all histopathological parameters [7]. Gleason score assessment according to ISUP 2005, using the most aggressive pattern as a secondary Gleason grade even when it is present in only a small area, yielded the best prognostic classification using groupings <7(4+3), 7(4+3)-8, and 9-10. In the present study, we examined whether the WGS in a single biopsy core would improve prognostic accuracy when compared with OGS. We also evaluated the prognostic value of compound Gleason score from the original pathology reports before the ISUP 2005 era.

# Methods

Material

The study was approved by the Ethical Committee of Tampere University Hospital (TAUH) and the National Authority for Medicolegal Affairs. From 1999 to 2003, 295 consecutive new prostate cancer patients, diagnosed from core biopsies, were primarily hormonally treated in the TAUH. Representative formalin-fixed, paraffin-embedded samples were available from 236 (80%) cases. Of these, clinical follow-up data were available for 233/236 (99%) cases. The end-point, biochemical progression, was defined as a ≥ 25% rise in PSA, with a PSA value ≥ 2.0 ng/ml above the nadir in two consecutive measurements, as recommended by The Prostate Cancer Clinical Trials Working Group (PCWG2) guidelines [8]. The median PSA value at the time of diagnosis was 15.5 ng/ml (mean 144 ng/ml, S.D. 772). Tumors were organ-confined (clinical T1-2) in 126 patients and advanced (cT3-4) in 107 patients. Bone scintigraphy was done in all symptomatic patients and in asymptomatic patients when PSA was ≥20 ng/ml or they had aggressive (original compound GS >7) prostate cancer. Based on bone scintigraphy, metastasis was detected in 40 (17%) patients. The primary hormonal treatments were luteinizing-hormone releasing-hormone (LHRH) analog (n=169), surgical castration (n=43), antiandrogen bicalutamide (n=21), and maximal androgen blockade (n=3).

Two slides from each patient were analyzed. The most representative hematoxylin and eosin (H&E)-stained slide, consisting of biopsies from the left or right lobe, was selected and scanned with Aperio ScanScope® XT (software version 9; Aperio Technologies, USA) and viewed in JPEG2000 format using JVSview virtual microscopy software (version 1.2) [9].

The WGS and OGS were evaluated according to the recommendations of the International Society of Urological Pathologists 2005 [3]. The overall Gleason score was derived as a sum of the

predominant and the most aggressive (or secondary) patterns of all the biopsy cores, treated as one long core. The worst Gleason score in a single biopsy core was assessed in cases for which one biopsy contained a higher Gleason grade (e.g., 4+4 cancer) and other cores a lower grade (e.g. 3+4). In the cases in which all positive biopsy cores contained same Gleason grade (e.g., 3+3) or there was only one core positive for cancer, the WGS was equal to the OGS. A Gleason score of 7 was considered as two separate grades (e.g., the WGS could equal 4+3 and the OGS 3+4). The CGS was obtained from the original pathology reports, in which it was assessed as sum of the predominant and the second most common Gleason patterns based on the evaluation of needle biopsy specimens from both lobes.

Statistical analysis

The agreement between Gleason scoring methods was analyzed with the κ-coefficient method. A survival analysis with PSA progression as end-point was performed using the Kaplan–Meier method, and the statistical significance of survival differences between patient groups was determined with a Mantel-Cox test. The univariate and multivariate Cox regression analyses were performed to calculate the relative risk estimates (RR) and to evaluate the independence of the prognostic grading methods.

# Results

Basic characteristics

The median age of the patients was 73.8 years (range 52.7-88.8). The median PSA at the time of diagnosis was 15.7 ng/ml (range 2.4-10750.0 ng/ml). The median follow-up time was 64.5 months (range 0-118). The average number of core biopsies from one lobe was 4.5 (median 4, range 1-9), and the average number of positive biopsy sites was 3.1 (median 3, range 1-7).

Needle biopsy findings

The number of cases with multiple positive biopsy sites was 191/236 (80.9 %) and WGS was higher than OGS in 43/236 (18.2 %) cases. In general, the modified GS system yielded to higher Gleason scores. The average GS was 7.6 (median 8, 95%-confidence interval 5.0-10.3) for WGS, 7.5 (7.0, 5.0-10.0) for OGS, and 7.0 (7, 4.5-9.6) for CGS. The distribution of Gleason scores according to grading method is shown in Figure 1. The number of cases with OGS=7 was 65. In 14 (22 %) cases of them there was at least one positive biopsy core containing higher-grade cancer (WGS 4+4=8). In 12 (31 %) of 39 cases with OGS 3+4=7, a positive biopsy core with the highest score showed WGS 4+3. Overall GS=9 was encountered in 52 cases of which the biopsy core with highest GS showed WGS=10 in 10 (19 %) cases. In three cases the difference between WGS and OGS was 2; in all of them OGS=8 (3+5 or 5+3) and WGS=10 (5+5).

Statistical analyses

The agreement between WGS and OGS was high (κ-coefficient=0.82). A significantly lower concordance was found between WGS and CGS (κ=0.48) and OGS and CGS (κ=0.44). All Gleason scoring methods provided prognostically highly significant information (Fig 2A, 2B, 2C, 2D, 2E, and 2F).

The univariate analyses of OGS and WGS yielded similar relative risks (Fig 2). Re-classification of the Gleason score groups to <7(4+3), 7(4+3)-8, 9-10 improved slightly prognostic value of the scoring. In the multivariate analysis of the six different Gleason grading methods, OGS reclassified as <7(4+3), 7(4+3)-8, 9-10 was the strongest (and only) independent prognostic factor (RR 2.6, 95% confidence interval 2.0-3.5).

## Discussion

The refinements of the ISUP 2005 consensus conference on Gleason scoring of needle biopsies has generally yielded better prognostic accuracy [10]. Our results indicate that modified Gleason scores according to the ISUP 2005 system are higher than compound GS´s from 1999-2003, and this upgrading is associated with improved prognostic accuracy. Moreover, the results suggest, that OGS may be a slightly stronger or at least equally adequate predictor of PSA progression than WGS, when assessed from pooled biopsies.

A major implication of the revised 2005 ISUP guidelines has been the integration of the most aggressive pattern into Gleason scores as a secondary pattern, even when the pattern is limited to a small area. Due to this, a fraction of cancers previously graded as GS 3+3=6 would nowadays end up with GS 3+4=7. It has been suggested that changing definitions shift the cut-off between low-grade and high grade cancers from 3+4 to 4+3 [11, 12]. The results of the present study are consistent with that.

According to the 2005 ISUP consensus conference, the highest (worst) GS should not be assessed from biopsies immersed in the same formalin container ("pooled biopsies") due to tissue fragmentation [3]. When all six biopsies from one lobe are formalin-fixed in the same container, they may become fragmented or overlap when embedded, disturbing the attempt to assess the WGS of the individual needle biopsies. On the other hand, WGS was recently shown to be a better predictor of the histopathological findings from subsequent radical prostatectomy specimens [4]. In our study, the WGS was assessed from the needle biopsies of one prostate lobe embedded in one paraffin block. Because of this, it is possible that our WGS results were biased by tissue fragmentation. However, in the majority of the cases (*n*=193/236, 82%), the WGS was equal to the OGS. If there was a bias due to fragmentation, we should expect more cases with WGS > OGS.

A major problem when multiple biopsies are stored in one container is that the exact locus information of the biopsies is lost unless site identifiers are used. The locus information is essential when considering targeted brachytherapy or cryotherapy in focal carcinomas. Moreover, the anatomic localization of carcinoma foci is useful when planning nerve-sparing radical prostatectomy and to avoid side effects from external-beam radiotherapy. The problems associated with placing multiple biopsies in one container can be overcome by immersing one core biopsy per formalin-container, which is quite laborious for all the participants: the urologist, laboratory technicians, and pathologist. Two major advantages of embedding multiple needle biopsy cores in one paraffin block are the reduced workload and the ability to analyze immunohistochemical stainings from all the biopsies at once, when deemed necessary.

There are a few limitations in the present study. First, although PSA progression works as a surrogate end-point for progressive prostate cancer, it does not necessarily correlate specifically with cancer or overall survival. Due to the small number of deaths in our series, we cannot conclude that OGS was a better prognostic factor in terms of death as a hard end-point. To address this question, a longer follow-up is needed. Second, CGS was not re-evaluated in the present study; instead it was obtained from the original pathology reports, which limits the value of this comparison. Third, the number of cases in which WGS > OGS was rather low (n=43).

## Conclusions

Overall and worst Gleason scores provide comparable prognostic information. We conclude that clinicopathological practice using one container per lobe (six biopsies) and yielding an overall Gleason score is a straightforward and cost-effective procedure that correlates well to prognosis in hormone-treated patients. Therefore, the use of individually embedded biopsies should be dictated

by the need for anatomic site information and weighed against the increased workload for the pathology laboratory

## Competing interests

The athors declare that they have no competing interests.

## Authors' contributions

This study has been designed by PK, TV, and TTT. The needle biopsy samples have been analyzed by TTT. Manuscript has been written by TTT, PK and JI. VT is responsible for the digitalization of the images and virtual microscope system. TLJT has acquired the clinical database of the patients. TV is responsible for the statistical analyses and finalization of the manuscript. Conclusions have been drawn mainly by TTT, JI, TV and PK.


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## Figure legends:

**Figure 1 – Gleason score distributions**

Distribution of Gleason scores (GS) according to the grading method. The number of cases with GS 7 is overemphasized by using compound GS, before the revised guidelines by ISUP 2005 were in routine use. Major changes between overall Gleason score (OGS) and worst Gleason score (WGS) are noted in shift from OGS 7 to WGS 8 and from OGS 9 to WGS 10.

**Figure 2- Survival curves**

Kaplan–Meier progression-free survival curves according to compound Gleason score (CGS) <7, 7, >7 from both lobes (A), CGS <7(4+3), 7(4+3)-8, 9-10 from both lobes (B), overall Gleason score (OGS) <7, 7, >7 from the most representative lobe (C), OGS <7(4+3), 7(4+3)-8, 9-10 from the most representative lobe (D), the worst Gleason score (WGS) <7, 7, >7 in a single biopsy from the most representative lobe (E), the WGS <7(4+3), 7(4+3)-8, 9-10 in a single biopsy from the most representative lobe (F). Relative risks (RR) with 95%-confidence intervals (95%-CI) according to Cox univariate analysis as well as p-values according to Mantel-Cox tests are shown.

**Figure 1.**

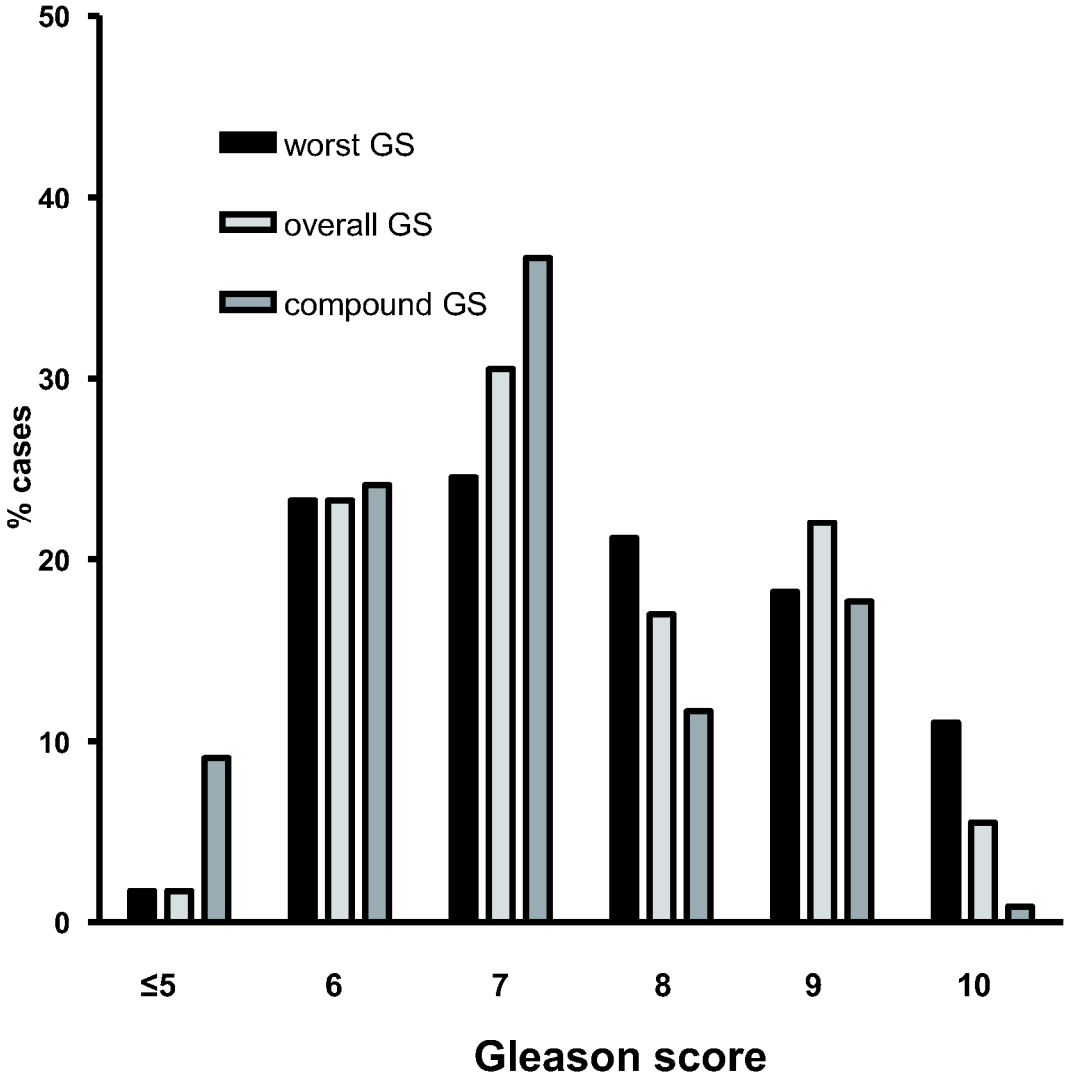
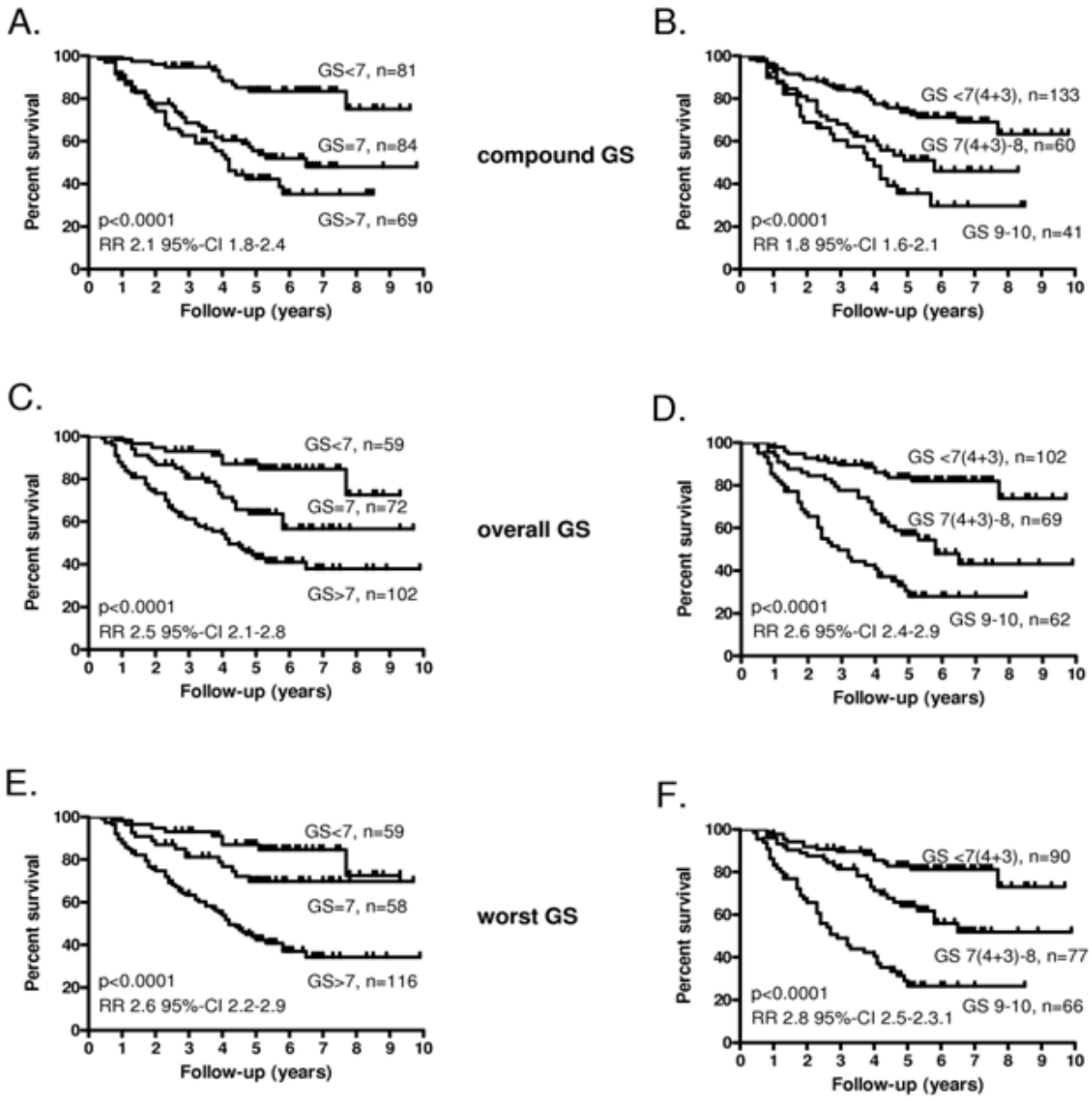

50
40
30
20
10
0
% cases
worst GS
overall GS
compound GS
≤5
6
7
8
9
10
Gleason score

**Figure 2.**


A.
Percent survival
GS<7, n=81
GS=7, n=84
GS>7, n=69
p<0.0001
RR 2.1 95%-CI 1.8-2.4
Follow-up (years)
compound GS
B.
Percent survival
GS <7(4+3), n=133
GS 7(4+3)-8, n=60
GS 9-10, n=41
p<0.0001
RR 1.8 95%-CI 1.6-2.1
Follow-up (years)
C.
Percent survival
GS<7, n=59
GS=7, n=72
GS>7, n=102
p<0.0001
RR 2.5 95%-CI 2.1-2.8
Follow-up (years)
overall GS
D.
Percent survival
GS <7(4+3), n=102
GS 7(4+3)-8, n=69
GS 9-10, n=62
p<0.0001
RR 2.6 95%-CI 2.4-2.9
Follow-up (years)
E.
Percent survival
GS<7, n=59
GS=7, n=58
GS>7, n=116
p<0.0001
RR 2.6 95%-CI 2.2-2.9
Follow-up (years)
worst GS
F.
Percent survival
GS <7(4+3), n=90
GS 7(4+3)-8, n=77
GS 9-10, n=66
p<0.0001
RR 2.8 95%-CI 2.5-2.3.1
Follow-up (years)